Novel Therapies in Oestrogen Receptor Positive Breast Cancer

Dr Farah Louise Lim

Submitted in partial fulfilment of the requirements of the Degree of MD(Res).

Statement of originality

I, Farah Louise Lim, confirm that the research included within this thesis is my own work or that where it has been carried out in collaboration with, or supported by others, that this is duly acknowledged below, and my contribution indicated. Previously published material is also acknowledged below. I attest that I have exercised reasonable care to ensure that the work is original and does not to the best of my knowledge break any UK law, infringe any third party's copyright or other Intellectual Property Right, or contain any confidential material. I accept that the College has the right to use plagiarism detection software to check the electronic version of the thesis. I confirm that this thesis has not been previously submitted for the award of a degree by this or any other university. The copyright of this thesis rests with the author and no quotation from it or information derived from it may be published without the prior written consent of the author.

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Details of collaboration and publications:

The OPPORTUNE trial was managed by the coordinating trial office at Brighton and Sussex Medical school (BSMS).

All tumour biopsies were reviewed centrally at Guys Hospital London and scanned onto the electronic database for subsequent review

Slides for IHC analysis were reviewed independently by Prof Sarah Pinder and Dr Louise Lim; as per trial design, the trial analyses and therefore data reported here were based on the geometric means of the two independent analyses

RNA analysis was performed at Genentech, Inc., South San Francisco, CA, US.

NGS analysis was performed at the Centre for Personalized Nanomedicine at the Australian Institute for Bioengineering and Nanotechnology, University of Queensland, Australia.

RPPA analysis was performed at Theranostics Health, Inc., Gaithersburg, MD, USA

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Data from the interim analysis (not detailed in this report) were presented at the San Antonio Breast Cancer Symposium 2013 and published in the Journal of Clinical Oncology (Schmid et al, J Clin Oncol 2015).

Preliminary data from biomarker analyses were presented at San Antonio Breast Cancer Symposium 2015

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List of abbreviations

AI	aromatase inhibitor
AIB1	amplified in breast cancer 1
AKT	AKT Serine/Threonine Kinase 1
AKT2	AKT Serine/Threonine Kinase 2
AP1	activator protein 1
BSMS	Brighton and Sussex Medical school
cfDNA	cell-free DNA
CRFs	case report forms
DCIS	Ductal Carcinoma in situ
DFS	disease-free survival
DNA-PK	DNA-dependent protein kinase
E	Oestrone
E2	Oestradiol
E3	Oestriol
EBCTCG	Early Breast Cancer Trialists' Collaborative Group
EGFR	Epidermal Growth Factor Receptor
ER	oestrogen receptor
EREs	oestrogen response elements
ESR1	Estrogen Receptor 1
ESR2	Estrogen Receptor 2
FGFR1	Fibroblast Growth Factor Receptor 1
FISH	fluorescence in situ hybridisation
FOXP3	Forkhead Box P3
FSH	follicle stimulating hormone
GATA4	GATA Binding Protein 4
GS	Gene Signature
H&E	haematoxylin and eosin
HER2/neu	human epidermal growth factor receptor 2
IGF1	Insulin Like Growth Factor 1
IHC	Immunohistochemistry
INPP4B	Inositol Polyphosphate-4-Phosphatase Type II B
Ki67	Marker Of Proliferation Ki67
LBD	ligand binding domain
LCIS	lobular carcinoma in situ
LH	luteinizing hormone
mTOR	Mechanistic Target Of Rapamycin Kinase
NF-κB	nuclear factor-κB
NGS	Next Generation Sequencing
p4E-BP1	Phosphorylated 4E-BP1
pAKT	phosphorylated AKT
PAX2	Paired Box 2
PDK1	Pyruvate Dehydrogenase Kinase 1

PI3K	Phosphoinositide 3-kinase
PIK3CA	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha
pPRAS40	phosphorylated PRAS40
PR	progesterone receptor
pS6	phosphorylated S6
PTEN	Phosphatase And Tensin Homolog
RFS	recurrence-free survival
RPPA	Reverse Phase Protein Arrays
RTK	receptor tyrosine kinase
SERD	selective ER down regulator
SERM	selective ER modulator
SP1	specificity protein 1
TLDU	terminal duct lobular unit
WOO	window of opportunity

Abstract

Background: There is increasing preclinical and clinical evidence that inhibition of the PI3K/mTOR pathway can improve the efficacy of endocrine treatment and overcome resistance, but patient stratification remains challenging due to the complex nature of the pathway which is characterised by multiple regulatory nodes and extensive crosstalk with other signalling pathways. Preoperative window studies are a validated clinical trial strategy to evaluate the impact of targeted therapies alongside endocrine agents in patients with early ER-positive breast cancer using the nuclear proliferation marker Ki67 as a surrogate endpoint of treatment benefit. The OPPORTUNE trial was designed to assess whether addition of the PI3K inhibitor Pictilisib can increase the anti-tumour effects of two-week preoperative anastrozole treatment in ER-positive breast cancer.

Aims: The 3 aims of this project were to

1. evaluate changes in tumour cell proliferation (by Ki67 expression pre and post-treatment) and apoptosis (by Caspase 3 expression) between patients treated with endocrine therapy alone and those treated with PI3K inhibitor Pictilisib plus Anastrozole.
2. characterise the subgroup of patients that derives the maximum benefit of addition of pictilisib with a focusing on the activation status of the PI3K pathway, using targeted next generation sequencing and gene expression analysis, and
3. to evaluate treatment-induced changes in molecular profiles between both treatment groups.

Results: We demonstrated that adding pictilisib to anastrozole significantly increased the anti-proliferative response compared anastrozole alone as measured by suppression of Ki67 expression. The rate of apoptosis however was low and there was no clear evidence of a treatment-associated increase in apoptosis.

When characterising the subgroup of patients that derives the maximum benefit of the addition of pictilisib, NGS identified patients with and without activating PIK3CA mutations but found no correlation between overall PIK3CA mutation status and added benefit of pictilisib. Our results suggested possible differences between helical and kinase domain PIK3CA mutations as patients with helical domain but not kinase domain mutations demonstrated a substantial benefit from pictilisib. Further assessment of PI3K pathway activation demonstrated an inverse association of a previously established PI3K inhibition gene signature with treatment response to anastrozole, suggesting this signature might be useful for selecting patients with partial endocrine resistance who might benefit from the addition of pictilisib. PAM50 analysis demonstrated that patients with Luminal B tumours but not patients with Luminal A tumours derive a benefit from PI3K inhibition.

The third aim of this project was to investigate treatment-associated changes in gene expression and protein expression and phosphorylation in the tumour and stroma. Using RNA and protein analysis, we were able to identify down-regulation of ER-mediated transcription and cell cycle progression, but found no differences in the expression of ER target genes between both study arms, suggesting that the preclinically observed induction of ER target genes by PI3K inhibition is not relevant in the context of combined endocrine and PI3K inhibitor therapy. Surprisingly, there

was no discernible differences between both groups in the expression and phosphorylation of PI3K downstream targets Phospho-AKT, pS6 and p4E-BP1.

Gene expression analysis furthermore demonstrated that short-term treatment with pictilisib and/or anastrozole has a modest impact on the tumour immune microenvironment but the potential clinical implications remain to be determined.

Conclusions: In summary, we were able to demonstrate that addition of the PI3K inhibitor pictilisib significantly increases the anti-proliferative response to anastrozole in ER-positive early breast cancers. By characterising PI3K activation and gene expression subtypes, we provided important information on the subgroup of patients who might benefit most from combined therapy which should guide optimal patient selection for future trials.

Table of Contents

Chapter 1 Introduction....14
1 Biology of Breast Cancer....14
2 HR-positive breast cancer....17
3 Endocrine therapy for breast cancer....19
Endocrine therapy for early breast cancer:....21
Endocrine therapy for metastatic breast cancer:....22
4 Endocrine resistance and co-targeting of the PI3K pathway....23
5 Clinical development of in inhibitors of the PI3K pathway in ER-positive breast cancer....29
6 Short term preoperative window studies....33
7 Rationale for the Opportune study....35
Chapter 2 Research aims and hypotheses....37
1 Establish the effect of the PI3K inhibitor pictilisib on tumour cell proliferation and apoptosis in patients with ER-positive breast cancer.....37
2 Evaluate the interaction between PI3K pathway activation and benefit from PI3K inhibition and the treatment effects of pictilisib in subgroups....37
3 Investigate treatment-associated changes in gene expression and protein expression and phosphorylation in the tumour and stroma....39
Chapter 3 Methods....40
1 Trial design....40
2 Tumour samples....44
3 Immunohistochemistry....44
4 DNA/RNA extraction....46
5 Gene expression analysis....46
6 Next Generation Sequencing....47
7 Reverse Phase Protein Arrays....48
8 Statistical Analysis....48
9 Summary of individual contribution....53
Chapter 4 Results....54
1 Effect of Study Treatment on Tumour Cell Proliferation....54
2 Effect of Study Treatment on Tumour Cell Apoptosis....61
3 Effect of study treatment in subgroups defined by PIK3CA mutations, Luminal A/B subtypes and baseline Ki67 scores....64

4 PI3K kinase pathway activation and treatment benefit.....73
5 Treatment-induced changes in gene/protein expression and phosphorylation 75
6 Effects of PI3K inhibition on the tumour microenvironment and immune system 82
Chapter 5 Discussion.....86
Chapter 6 References.....99

List of Figures

Figure 1 Mode of action of oestradiol (Wakeling, 2000). 18
Figure 2 Strategies for endocrine therapy of breast cancer and main classes of hormone therapy. 20
Figure 3 Mode of action of SERMs such as tamoxifen and SERDs such as fulvestrant (Wakeling, 2000). 20
Figure 4 Mechanisms of Endocrine Resistance. 25
Figure 5: PI3K-AKT-mTOR pathway. 27
Figure 6 A simplified view of a window-of-opportunity trial. 34
Figure 7: OPPORTUNE trial design. 41
Figure 8: Trial consort diagram 54
Figure 9: Comparison of the two independent Ki67 analyses at baseline (a) and at EOT (b) 56
Figure 10: Individual Ki67 changes from baseline to Day 15 and Anti-proliferative response to study treatment 59
Figure 11: Relationship between Ki67(%) and apoptosis (%) before and after 2 weeks of treatment irrespective of treatment arm. 62
Figure 12: Mean percentage Growth Index suppression 63
Figure 13: Anti-proliferative response to study treatment by PIK3CA mutation status; e9: exon 9 domain mutations (helical domain); e20: exon 20 domain mutations (kinase domain) 66
Figure 14: A) Somatic variant analysis and response to anastrozole or anastrozole/pictilisib. B) Forest plots 68
Figure 15: Anti-proliferative response to study treatment; A) anti-proliferative response by Luminal subtype defined by PAM50; B) anti-proliferative response by Luminal subtype defined by baseline Ki67 expression (cut-off 14%) 70
Figure 16: Anti-proliferative response to study treatment PR status (a) and tumour grade (b) 71
Figure 17: PI3K mutation and PI3K inhibitor sensitivity gene signatures: a) association of baseline PIK3 inhibitor sensitivity GS (O'Brien) score and Luminal B phenotype; b) down-regulation of post-treatment PIK3 inhibitor sensitivity GS (O'Brien) scores in both treatment arms; c) post-treatment PIK3CA mutation-associated GS (Loi) in both treatment groups; A=anastrazole only, C=combination with pictilisib. 74
Figure 18 Differentially-expressed genes between pre- and post-treatment samples in the anastrozole arm (a) and anastrozole and pictilisib arm (b) 76
Figure 19: Treatment-induced changes in expression of ER target genes PR and GREB1; A, anastrozole alone; C, combination. 78
Figure 20: RPPA analysis of Phospho-AKT levels (a) and pS6 levels (b) after treatment with anastrozole or combination therapy; treatment-associated upregulation of PIK3CA-regulated genes PIK3IP1 and IRS2 80
Figure 21: RPPA analysis focusing on key genes involved in the activation of the PI3K pathway and cell cycle. A) Mean end-of-treatment RPPA expression in the anastrozole and combination therapy groups; B) Mean treatment-associated changes in RPPA expression with anastrozole and anastrozole plus pictilisib 81
Figure 22 a) Impact on markers of immune cell populations (CD68, CD4, CD8A) in the post-treatment samples; b) Treatment effect on T_{eff} and T_{reg} signatures; c) APC immunosuppressive signature; d) T-cell immune-suppressive signature 85

List of tables

Table 1 Randomised trials of inhibitors of PI3K, AKT and/or mTOR in metastatic breast cancer. 30
Table 2 Study Objectives and Endpoints 49
Table 3 Patient demographics and tumour characteristics at baseline 55
Table 4 Anti-proliferative response to anastrozole or anastrozole plus pictilisib; 60
Table 5 Induction of apoptosis with anastrozole or anastrozole plus pictilisib; 61
Table 6 Treatment-associated change in growth index (GI), 63
Table 7 PIK3CA status and anti-proliferative response to anastrozole or anastrozole plus pictilisib 65
Table 8 The top 10 gene transcripts overexpressed and repressed in A) anaztrozole and B) combination treated patients. 77

Chapter 1 Introduction

1 Biology of Breast Cancer

Breast cancer remains the most common malignancy affecting women in Europe or North America, corresponding to an age-corrected annual incidence of 100 to 120 per 100000 females. Oestrogen and the oestrogen receptor (ER) play an important role in the development and progression of breast cancers. Breast cancer is a complex disease and presents a multifactorial aetiology. There are a wide range of risk factors from age, lifestyle, and diet to family history and genetic and epigenetic alterations in the genome. The nature of the cancer significantly influences prognosis and risk of recurrence ("Breast cancer incidence (invasive) statistics | Cancer Research UK," n.d.)

The normal breast is made up of a system of branched epithelial tubes, called ducts, which connect the lobules to the nipple where milk is secreted. The lobules contain secretory units called acini. The ducts of the breast are lined by a single layer of epithelial cells, surrounded by a layer of myoepithelial cells or basal cells, which are encircled by a basement membrane made up of laminin and collagen. This whole structure is surrounded by connective tissue and embedded into adipose tissue (Ali and Coombes, 2002). Breast cancers arise from the mammary epithelium and most commonly the epithelial cells of the distal ducts or the lobules. Breast cancer development is thought to begin with a benign epithelial lesion characterised by abnormal structure of the breast duct or lobule. This evolves into atypical hyperplasia where an extra cell layer grows within the lumen. These cells grow and proliferate

into an early stage in situ carcinoma. This develops into invasive breast cancer with cells reaching towards the nearest blood vessels and then lymph vessels to cause metastatic spread (Hu et al., 2008; Mego et al., 2010).

Breast cancer can be broadly divided into in situ carcinoma and invasive carcinoma. Invasive breast cancer describes a group of malignant cancers, which have invaded into the surrounding breast tissue and have the potential to metastasise. Histological classification is not sufficient to fully describe the heterogeneity of invasive breast cancers. However, advances in molecular understanding along with gene expression profiling has helped refine breast cancer classification. Such classification is especially relevant when considering the medical treatment for breast cancers.

For many years, breast cancers have been divided into 3 subgroups; based on presence of the 2 hormone receptors (HR), ER and progesterone receptor (PR), and the human epidermal growth factor receptor 2 (HER2/neu) (Yersal and Barutca, 2014):

- HR-positive, HER2-negative breast cancer, which express ER and/or PR but are negative for HER2,
- HER2-positive breast cancer, where HER2 is over-expressed or amplified, regardless of ER and/or PR expression, and
- Triple Negative Breast cancer, which are negative for ER, PR and HER2.

More recently, gene expression profiling has reshaped our understanding of breast cancer by defining four intrinsic molecular subtypes, including the two ER-positive subtypes luminal A and luminal B, as well as a HER2-enriched and a basal-like

subtype. This classification has significant clinical relevance especially with regards the luminal subtypes.

The luminal subtypes share expression of the ER and/or PR and generally have a better outcome than the HER2 and basal-like subtypes (Glueck et al., 2013; Koboldt et al., 2012; Sorlie et al., 2003). Luminal A breast cancers account for approximately 40% of breast cancers and are characterised by high ER-signalling and the absence of overexpression or amplification of HER2. Luminal A cancers are generally low-grade tumours with a relatively low proliferation rate, excellent response to endocrine therapy and a favourable prognosis (Voduc et al., 2010).

Luminal B cancers, which correspond to approximately 20% of cases, tend to have lower ER and/or PR expression, expression of proliferation markers, and higher histologic grade, which correlates with a worse prognosis and higher tendency to relapse compared to Luminal A tumours (Ignatiadis and Sotiriou, 2013; Sorlie et al., 2003). Luminal B cancers also exhibit partial endocrine resistance. Luminal A and Luminal B cancers should therefore be regarded as distinct entities with specific oncogenic drivers, rather than more proliferative varieties of the same tumour subtype.

HER2+ represents 20-30% of all diagnosed breast cancers, and exhibit over expression of HER2, with under-expression of luminal-associated genes (Révillion et al., 1998). Basal-like tumours account for up to 15% of all breast cancers. These tumours primarily exhibit no expression of ER, PR or HER2, but are a highly heterogeneous sub-group of breast cancers (Sinn and Kreipe, 2013; Viale, 2012) and have been sub divided in up to 6 separate subtypes (Lehmann et al., 2011). The highly

diverse nature of breast cancer, and increasing understanding of molecular markers and genetic profiling, means subtyping and classification is subject to continuous changes and adjustments.

2 HR-positive breast cancer

The majority of breast cancers are HR-positive and endocrine therapy constitutes the key treatment for these patients (Early Breast Cancer Trialists' Collaborative Group (EBCTCG) et al., 2015).

The ERs are members of the nuclear hormone–receptor superfamily which includes receptors for other steroid hormones, thyroid hormone, vitamin D, and retinoic acid. There are two different ERs, ERα and ERβ, which are encoded by the two genes ESR1 and ESR2 (Green et al., 1986; Kuiper et al., 1996). Whereas ERα is clinically highly relevant for breast cancer, the function of ERβ remains still unclear.

Classically, the ERs function as transcription factors in the nucleus when they are bound to their respective ligands (Figure 1). ERs contain several functional domains, including the DNA-binding domain, a dimerization domain, a ligand-binding domain and several transcription activating domains. After entering cells, the ligand oestradiol (E2) binds to the ER, leading to a conformational change of the receptor that facilitates dissociation of receptor-associated proteins such as heat-shock protein (HSP) 90, homo-dimerization and association with co-regulatory proteins, such as amplified in breast cancer 1 (AIB1), that regulate the transcription of oestrogen-responsive genes (McKenna et al., 1998; Osborne et al., 2001). The dimerised ER binds to small palindromic DNA motifs known as oestrogen response elements (EREs) in the promoters of oestrogen-response genes. Two distinct

activation domains, AF1 and AF2, have been shown to mediate the activity of the ER with transcriptional co-activators or co- repressors. Whilst AF2 is integral to the ligand-binding domain and therefore ligand-dependent transcription, AF1 activity is regulated by phosphorylation which can occur through multiple cellular kinases. Both AF1 and AF2 can activate transcription independently and/or act synergistically. In addition to the classical nuclear transcriptional regulation, non-classical regulation at non-ERE sites and non-genomic ER activity have been described, which can play a relevant role in endocrine resistance.

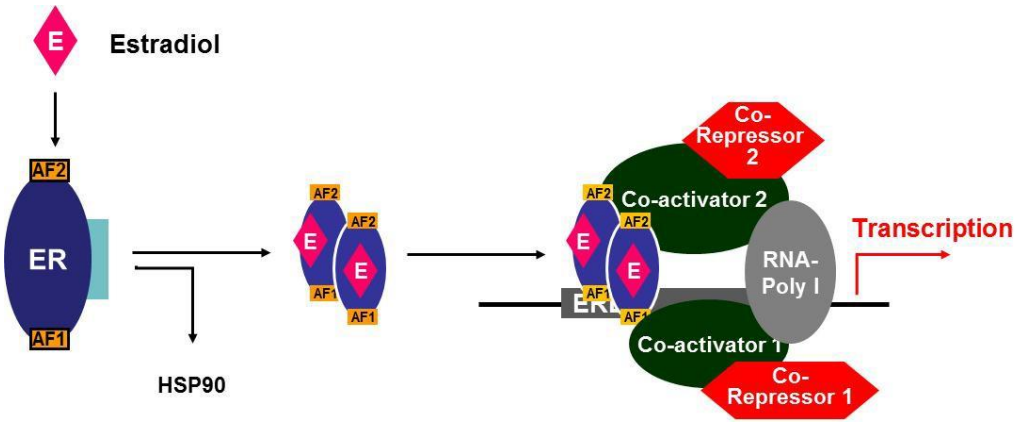


Figure 1 Mode of action of oestradiol (Wakeling, 2000). After entering cells, E2 binds to the ER, leading to a conformational change of the receptor that facilitates homo-dimerization and association with co-regulatory proteins, that regulate the transcription of oestrogen-responsive genes. The dimerised ER binds to the EREs in the promoters of oestrogen-response genes to regulate target gene transcription. ER, Oestrogen receptor, E, Oestradiol, AF, activation function, ERE, oestrogen response element.

The naturally occurring oestrogens 17β-oestradiol (E2), which is the most abundant circulating form, oestrone (E), and oestriol (E3) are C18 steroids derived from cholesterol. In premenopausal women, oestradiol (E2) is synthesized primarily in the ovaries. Ovarian function and E2 synthesis are regulated by the pituitary gonadotropins, FSH and LH. Oestrone and E3 are primarily formed in the liver from E2. Additional sources of E2 synthesis are liver, fat tissue and muscles, which are the

main sources for the non- reproductive functions of E2 in postmenopausal women. In addition, breast tumours can also produce E2 which is one of the reasons that intra-tumoral concentrations of E2 can be more than 20-fold higher than those present in the plasma.

3 Endocrine therapy for breast cancer

There are several ways of blocking the effects of oestrogen in ER-positive breast cancer (Figure 2). The main endocrine treatments strategies are directed at

- inhibiting the action of ER in breast tissue using selective ER modulators (SERMs) (such as tamoxifen), which bind to the ER competing with E2,
- withdrawing oestrogen through aromatase inhibitors (AIs; such as anastrozole, exemestane) which block peripheral oestrogen synthesis, or by ovarian ablation, or
- targeting ER for degradation with selective ER down regulators (SERDs; fulvestrant).

SERMs and SERDs are also often classified as anti-oestrogens based on the fact that both types of agents directly bind to the ER. There are distinct differences in the mode of action between SERMs and SERDs. The SERMs inhibit AF2- but not AF1-activation (Figure 3). Because the ER activity in breast cancers is mainly due to AF2, SERMs act as an antagonist in breast cancer. However, in other tissues such as the uterus, AF1 activity can be more significant, resulting in greater agonistic activity of tamoxifen. SERDs such as fulvestrant prevent activation of both AF1 and AF2 and also lead to increased degradation of the ER; SERDs are therefore anti-oestrogenic in all tissues (Figure 3).

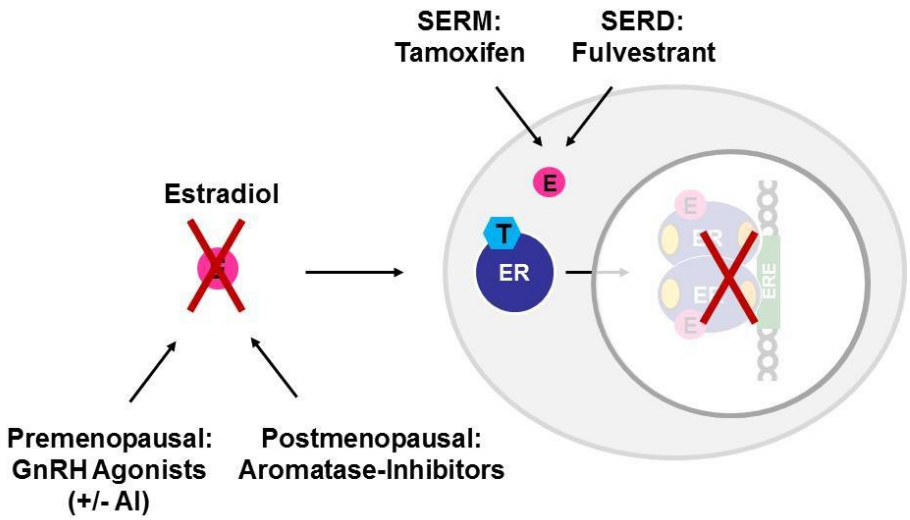


Figure 2 Strategies for endocrine therapy of breast cancer and main classes of hormone therapy. The main endocrine treatments strategies are directed at reducing the production of E2 or at blocking the effect of E2 at the ER. ER, Oestrogen receptor, E, Oestradiol, SERM, selective ER modulators; SERD, selective ER down regulators, T, Tamoxifen, ERE, oestrogen response element.

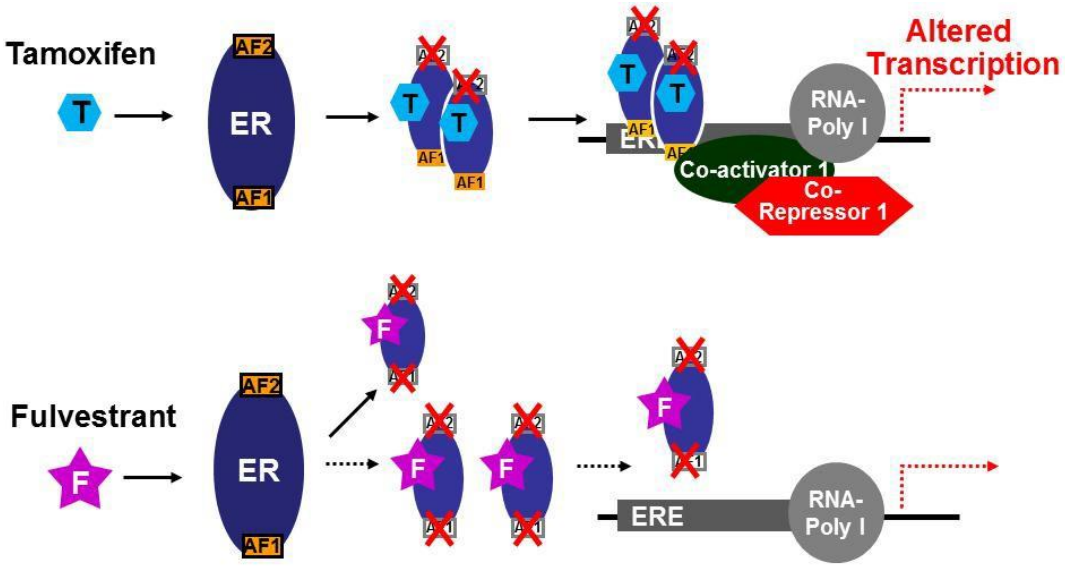


Figure 3 Mode of action of SERMs such as tamoxifen and SERDs such as fulvestrant (Wakeling, 2000)*. After entering cells, Tamoxifen binds to the ER, leading to a conformational change of the receptor that allows homo-dimerization and altered association with co-regulatory proteins but inhibits AF2 (whilst retaining AF1-activation); Fulvestrant effectively stops homo-dimerization and association with co-regulatory proteins, thus blocking activation of both AF1 and AF2 and also leading to increased degradation of the ER. ER, Oestrogen receptor, E, Oestradiol, AF, activation function, ERE, oestrogen response element; T, Tamoxifen, F, Fulvestrant.*

Endocrine treatment is generally offered to all patients with ER-positive early breast cancer, and it is also the first treatment option to most women with hormone-sensitive, HER2-negative metastatic breast cancer. This recommendation is based upon lower toxicity of endocrine treatment and often longer durations of response in this subset as compared with cytotoxic chemotherapy, with no difference in overall survival (Early Breast Cancer Trialists' Collaborative Group (EBCTCG) et al., 2015).

Endocrine therapy for early breast cancer: In early breast cancer, tamoxifen, alone or in combination with ovarian suppression, remains a standard for premenopausal patients, whereas AIs are now generally considered standard for postmenopausal women. This was based on a big meta- analysis carried out by the Early Breast Cancer Trialists' Collaborative Group (EBCTCG) including individual data of >30,000 postmenopausal women with ER-positive early breast cancer treated within randomised trials AI versus tamoxifen. Primary outcome measures used were any recurrence of breast cancer, breast cancer mortality, death without recurrence, and all-cause mortality. Results showed that AIs reduce the recurrence rates of breast cancer by about 30% compared to tamoxifen and the 10-year breast cancer mortality rates by about 15% (Early Breast Cancer Trialists' Collaborative Group (EBCTCG) et al., 2015). AIs are also increasingly used in premenopausal women but require combination with ovarian suppression in this group of patients in order to be active.

Most of the experience with endocrine therapy is for 5 years. However, more recently, several trials have evaluated the benefit of Tamoxifen or AIs beyond 5 years of therapy, as well as the benefits of 5 years AI after either 2-3 years of Tamoxifen or after 5 years of Tamoxifen. Randomised trials have demonstrated increased overall

survival and distant disease-free survival (DFS), reduced breast cancer-specific mortality, a decreased risk of recurrence, and a decreased risk of contralateral breast cancer associated with extended endocrine therapy. This has to be seen in context with an increased risk of endometrial cancer (if continuing tamoxifen), hot flashes and other menopausal symptoms, deep vein thrombosis or pulmonary embolism (tamoxifen), ischemic heart disease (AI), osteopenia/osteoporosis (AI), and uterine cancer (tamoxifen).

Endocrine therapy for metastatic breast cancer: In metastatic breast cancer, single agent endocrine therapy used to be the standard until recently, but is increasingly replaced by combination therapies, e.g. with CDK4/6 inhibitors, mTOR inhibitors or PI3K inhibitors (see below).

In terms of single agent endocrine therapies, there is no universal standard for the optimal sequence. In patients who received adjuvant treatment with Tamoxifen, third generation AIs are often considered first line treatment of choice for metastatic disease as they have produced a significant survival advantage compared with tamoxifen, progestogens and non-specific AIs in randomised clinical studies (Early Breast Cancer Trialists' Collaborative Group (EBCTCG) et al., 2015). However, recent data from two randomised trials suggest that Fulvestrant might be slightly more effective than AIs in patients who have not received prior endocrine therapy. In the randomised phase 2 trial FIRST, patients treated with Fulvestrant had a significantly longer median overall survival of 54.1 months compared to 48.4 months in the AI treated arm (Ellis et al., 2015). This was confirmed in a subsequent randomised phase 3 trial of fulvestrant versus anastrozole (FALCON trial), demonstrating a significantly

longer progression-free survival in the fulvestrant group than in the anastrozole group (hazard ratio [HR] 0·797, 95% CI 0·637–0·999, p=0·0486). The median progression-free survival was 16·6 months (95% CI 13·83–20·99) in the fulvestrant group versus 13·8 months (11·99–16·59) in the anastrozole group (Robertson et al., 2016).

Patients who progress on AIs can be considered for tamoxifen and/or Fulvestrant or for a steroidal AI if they received a non-steroidal AI (exemestane) in the previous line. Initial trials with fulvestrant failed to show a significant difference compared to exemestane or tamoxifen, but subsequent studies showed that higher doses of fulvestrant might increase the activity.

4 Endocrine resistance and co-targeting of the PI3K pathway

Despite the fact that the majority of patients with ER-positive breast cancer initially benefit from endocrine therapy, all patients with metastatic disease will eventually experience endocrine resistance. Endocrine resistance remains one of the most challenging clinical problems and substantial efforts of preclinical and clinical research is being directed at elucidating the processes of resistance.

Multiple studies indicate that acquired resistance to endocrine therapy is a progressive, step-wise phenomenon which transforms breast cancer cells from an oestrogen-dependent phenotype that is responsive to endocrine therapy, to a non-responsive phenotype, and eventually to an oestrogen-independent phenotype (Giuliano et al., 2011).

From a molecular point of view, two main categories of endocrine resistance can be differentiated, characterised by either altered ER signalling leading to ligand-independent ER activation or an increase in alternative, hormone-independent mitogenic or survival pathways (Figure 4). There is substantial overlap between both categories mainly through phosphorylation of the ER and co-regulatory proteins through alternative intracellular signalling pathways, leading to constitutive, ligand-independent ER activation (Johnston, 2015).

In the past few years, several studies have revealed the presence of acquired mutations in ESR1 that confer ligand-independent and constitutive activation. Whilst ESR1 mutations are extremely rare in early breast cancer, they are commonly found in metastatic breast cancer with an increasing incidence over time, especially in AI-treated patients, reaching up to 40% (Fribbens et al., 2018, 2016; Jeselsohn et al., 2015; Robinson et al., 2013; Toy et al., 2013). These mutations tend to segregate around a 'hotspot' area within the ligand binding domain (LBD) of the ER, clustering between amino acids 534–538, though mutations at other positions including S463 and E380 have also been described (Carlson et al., 1997; Li et al., 2013). Multiple lines of preclinical research demonstrate that these mutations are constitutively active conferring constitutive, ligand-independent activation. ER LBD mutations may thus account for acquired endocrine resistance in a proportion of patients with metastatic breast cancer, with the idea being that as a patient becomes exposed to more and more endocrine treatment, this selective pressure results in clonal expansion of these mutant clones which in turn results in resistance (Jeselsohn et al., 2015).

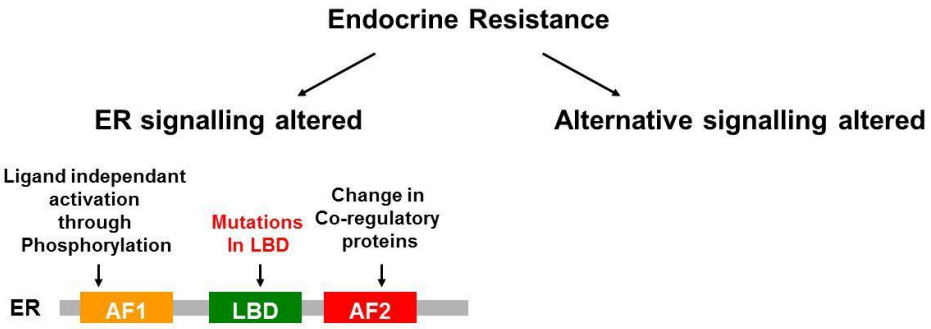


Figure 4 Mechanisms of Endocrine Resistance. Endocrine resistance result from altered ER signalling via cross-phosphorylation, mutations in the LGB or changes in co-regulatory proteins, or through an increase in alternative, hormone-independent mitogenic or survival pathways. ER, Oestrogen receptor; AF, activation function, LBD, Ligand binding domain.

Upregulation of intracellular proliferation and cell survival signalling pathways is widely considered a key resistance mechanism, leading to alternative, ER-independent pathways for proliferation and survival. Such adaptive mechanisms can result from genetic or epigenetic changes within the tumour that drive hormone-independent mitogenic pathways. The main pathways implicated in this process are CDK4/6-Cyclin D signalling and the PI3K/AKT/mTOR pathway.

Since this MD project was started, therapeutic targeting the Cyclin-dependent kinase 4/6 (CDK4/6) pathway has become a new standard for the treatment of ER-positive breast cancer. Three CDK4/6 inhibitors (palbociclib, ribociclib and abemaciclib) are currently approved for the use in HR-positive, metastatic breast cancer, either in combination with AIs or with Fulvestrant. This was based on several randomized trials demonstrating consistently that the addition of CDK4/6 inhibitors to endocrine therapy substantially improves progression-free survival with HRs in the range of 0.52-0.58 (Cristofanilli et al., 2016; Finn et al., 2016; Goetz et al., 2017; Hortobagyi et al., 2016b; Sledge et al., 2017; Turner et al., 2015).

Targeting the PI3K/AKT/mTOR pathway: In addition to the CDK4/6 pathway, targeting the PI3K/AKT/mTOR pathway remains a key therapeutic strategy. This is based on substantial preclinical and clinical evidence that aberrant signalling through the Phosphoinositide 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) signalling pathway plays a critical role in endocrine resistance (Miller et al., 2011).

The PI3K/AKT/mTOR pathway functions as a sensor of mitogen, energy and nutrient levels and is a central controller of cell growth and survival (Figure 5). The PI3K/AKT/mTOR pathway is highly conserved and generally tightly controlled. The PI3K is directly activated through trans-membrane receptors which trigger PI3K-catalysed conversion of phosphatidylinositol (3,4)-bisphosphate (PIP2) lipids to phosphatidylinositol (3,4,5)-trisphosphate (PIP3). Binding of PIP3 to AKT at the plasma membrane leads to AKT activation and subsequent downstream activation of mTOR by phosphorylating and inactivating PRAS40 and TSC2. The mTOR kinase is the catalytic component of two distinct multiprotein complexes, mTORC1 and mTORC2, which are defined by their associated proteins. Both complexes have different cellular functions. mTORC1 activates p70S6K and 4EBP1, which in turn activate the ribosomal protein S6 and eIF4E, promoting protein synthesis and cellular proliferation.

The PI3K/AKT/mTOR pathway is one of the most frequently activated pathways in human tumours. Aberrant PI3K pathway activation occurs in approximately 50% of ER-positive breast cancer, most commonly through activating mutations of the PI3K catalytic subunit (Bärlund et al., 2000; Bellacosa et al., 1995; Feilotter et al., 1999; Samuels et al., 2004). Approximately 40% of ER-positive breast cancer patients have

activating somatic mutations in PIK3CA, most frequently in the hotspots exons 9 and 20 of PIK3CA, the gene that encodes the p110α isoform of PI3K (Baselga et al., 2017; Cancer Genome Atlas Network, 2012; Hortobagyi et al., 2016a). An additional 5-10% have loss of the negative regulator PTEN or activating mutations of AKT1. Additional pathway aberrations such as amplification or mutations of the effectors AKT1, AKT2, or PDK1, or of upstream receptor tyrosine kinases such as HER2, EGFR or FGFR1, or loss of the negative regulator INPP4B, have been described in ER-positive breast cancer, making breast cancer a rational target for PI3K inhibitors.

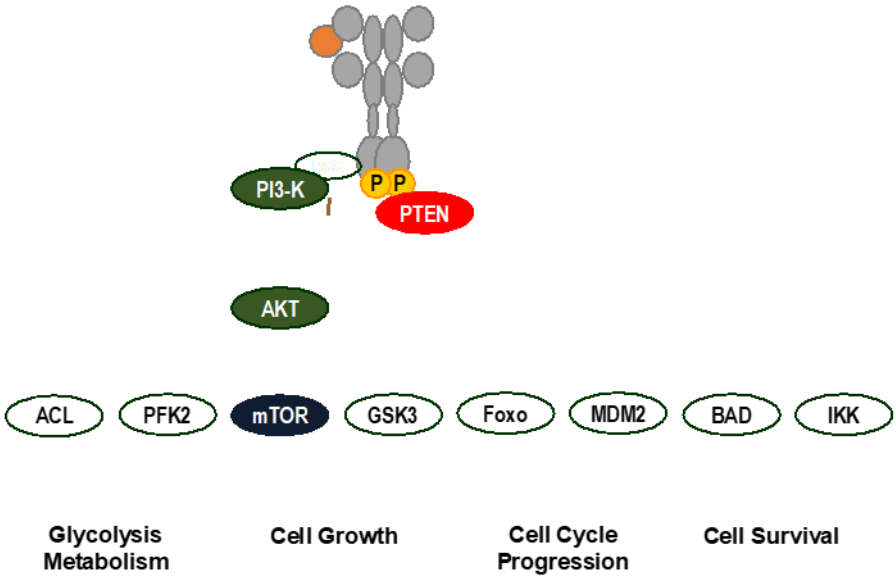


Figure 5: PI3K-AKT-mTOR pathway. The PI3K is directly activated through trans-membrane receptors which trigger PI3K-catalysed conversion of phosphatidylinositol (3,4)-bisphosphate (PIP2) lipids to phosphatidylinositol (3,4,5)-trisphosphate (PIP3). Binding of PIP3 to AKT at the plasma membrane leads to AKT activation and subsequent downstream activation of mTOR, promoting protein synthesis, cellular proliferation and survival. P, Phosphorylation.

Activation of the PI3K pathway has been demonstrated to promote resistance to endocrine therapy and hormonal independence in ER-positive breast cancer models

(Miller et al., 2010, 2009; Shou et al., 2004). Proteomic and transcriptional profiling of human HR-positive tumours suggest that increased signalling through the PI3K/AKT/mTOR pathway is associated with lower ER levels and resistance to endocrine therapy (Creighton et al., 2010; Miller et al., 2010). Inhibition of the PI3K/mTOR pathway in non-clinical models has been shown to upregulate ER/PR expression (Creighton et al., 2010) and enhance the antitumor effect of letrozole (Boulay et al., 2005).

There is increasing preclinical and clinical evidence that inhibition of the PI3K/mTOR pathway can improve the efficacy of endocrine treatment and overcome resistance (Baselga et al., 2012; Boulay et al., 2005; Crowder et al., 2009; Ghayad et al., 2010; Thorpe et al., 2014). Importantly, oestradiol can suppress apoptosis induced by PI3K inhibition in ER-positive breast cancer, suggesting that PI3K-dependent and oestradiol-dependent cell survival mechanisms are independent (Crowder et al., 2009). Preclinical studies demonstrate additive/synergistic anticancer activity and in some cases synthetic lethality of PI3K, AKT and/or mTOR inhibition and oestrogen deprivation, providing a strong rationale for the combination of PI3K/mTOR inhibitors and endocrine therapy (Boulay et al., 2005). It has furthermore been shown that PIK3CA wild-type or mutant tumours equally benefit from combined PI3K/Akt/mTOR pathway inhibition and endocrine therapy, suggesting that eligibility in clinical trials should not be restricted by PIK3CA mutation status. This, combined with the association of therapeutic resistance with increased PI3K pathway signalling, suggests that inhibition of PI3K/Akt/mTOR signalling could have broad applications in the treatment of breast cancer.

5 Clinical development of in inhibitors of the PI3K pathway in ER-positive breast cancer

The PI3K/Akt/mTOR has been targeted on several levels. Table 1 provides and overview on pivotal clinical trials of PI3K, mTOR or AKT inhibitors targeting in metastatic ER-positive breast cancer.

In a first randomised phase 2 trial in postmenopausal women with ER positive metastatic breast cancer (MBC), the addition of the mTOR inhibitor everolimus to tamoxifen showed a significant improvement of the time to progression (8.6 months vs 4.5 months, p=0.02) and overall survival (median not reached vs 24.4 months, p=0.01) compared to Tamoxifen alone (Bachelot et al., 2012). This was confirmed in a subsequent phase 3 study, BOLERO -2, which showed that the addition of everolimus to exemestane more than doubled progression free survival (PFS) compared to single agent exemestane in MBC patients whose disease was refractory to previous AI therapy (median PFS was 6.9 months with everolimus vs 2.8 months with placebo (Baselga et al., 2012). Interestingly, the benefit of everolimus was demonstrated irrespective of the presence of activating PIK3CA mutations (Hortobagyi et al., 2016a). In a randomised phase II study of neoadjuvant treatment in patients with ER positive breast cancer, everolimus increased the efficacy of letrozole as measured by a decrease in Ki67 expression, and improved the clinical response rate (Baselga et al., 2009).

In contrast to the everolimus studies, trials with the mTOR inhibitor temsirolimus failed to demonstrate a benefit. In the HORIZON trial, AI-naive MBC patients were

treated with the mTOR inhibitor temsirolimus in addition to letrozole. The trial failed to show an improvement in the primary end point PFS (median, 9 months; HR, 0.9; 95% CI, 0.76 to 1.07; p=0.25); one of the possible explanations might be that PI3K/mTOR pathway activation may be more relevant in acquired resistance (Wolff et al., 2013). Similarly, the TORC1/2 inhibitor vistusertib failed to demonstrate a benefit when added to fulvestrant in MBC, possibly due to insufficient inhibition of TORC1 activity (Schmid et al., 2019).

Trial	Class	Treatment Arm	Number of Patients	Median PFS	HR/ P-value
TAMRAD	mTOR	Eve + Tam Tam	54 57	8.6 4.5	0.54/ 0.002
BOLERO-2	mTOR	Eve + Exe Exe	485 239	7.8 3.2	0.38/ <0.0001
HORIZON	mTOR	Let + Tem Let	556 556	9.0 8.9	0.9/ 0.25
FERGI	Pan PI3K	Fulv + Pict Fulv	89 79	6.6 5.1	0.74/ 0.096
BELLE-2	Pan PI3K	Fulv + Bupa Fulv	576 571	6.9 5.0	0.78/ 0.00021
BELLE-3	Pan PI3K	Fulv + Bupa Fulv	289 143	3.9 1.8	0.67/ 0.0003
SOLAR1	a-PI3K	Fulv + Alpe Fulv	169 172	11.0 5.7	0.65/ <0.001
SANDPIPER1	a-PI3K	Fulv + Tase Fulv	340 176	7.4 5.4	0.70/ 0.0037
FAKTION	AKT	Fulv + Capi Fulv	69 71	10.3 4.8	0.58/ 0.0044
MANTA	mTOR/ TORC1/2	Fulv + Eve Fulv + Vist Fulv	45 81 57	12.3 7.6 5.4	 0.88/0.46 0.63/0.01

Table 1 Randomised trials of inhibitors of PI3K, AKT and/or mTOR in metastatic breast cancer. Eve, Everolimus; Exe, Exemestane, Tem, Temsirolimus; Alpe, Alpelisib; Tase, Taselisib; Capi, Capivasertib; Vist, Vistusertib pan PI3K, pan PI3K inhibitor; a-PI3K, alpha-specific PI3K inhibitor,

The initial MBC clinical development of PI3K inhibitors focused on pan-PI3K inhibitors. As preclinical data were inconclusive towards whether PIK3CA mutations are predictive of response or benefit with PI3K inhibitors, most pan-PI3K inhibitor trials included patients irrespective of their PIK3CA mutations status.

In the randomised phase 2 FERGI study, which looked at the addition of the PI3-kinase inhibitor pictilisib to fulvestrant versus fulvestrant alone in patients with ER-positive, AI-resistant MBC, addition of the pan PI3K inhibitor Pictilisib to fulvestrant failed to show a significant improvement in PFS. There was no association of potential treatment benefit of pictilisib with the presence of PIK3CA mutations (Krop et al., 2016). A more recent phase 3 trial with the pan-PI3K-inhibitor buparlisib (BELLE2) (Pritchard et al., 2013) showed a small but significant difference in median PFS for the combination of buparlisib with fulvestrant (5 months versus 6.9 months, p <0.001) compared to the fulvestrant alone in MBC patients with previous AI therapy. A similar relative benefit was observed in the BELLE-3 trial, which included MBC patients after prior mTOR inhibitor therapy (Di Leo et al., 2018).

As in FERGI, the presence of PIK3CA mutations in the primary tumour was not predictive in the BELLE studies for the benefit of the PI3K inhibitor. However, analysis of PIK3CA mutations from plasma-derived cell-free DNA (cfDNA) suggested an increased benefit of buparlisib in PIK3CA mutant tumours (7 months vs 3.2 months, p<0.001). This is somewhat surprising as several studies have demonstrated high concordance of PIK3CA mutations measured in primary tumours and subsequent cfDNA analysis. Despite the substantial pre-clinical activity, the results from these two randomised clinical trials of pan PI3K inhibitors have been relatively

disappointing, compared to the activity of everolimus. One of the potential explanations might lie in the substantial toxicity of pan PI3K inhibitors. Many patients in the FERGI and BELLE studies required dose reductions or discontinued treatment with the PI3K inhibitors due to toxicity, potentially limiting its efficacy. In the BELLE-2 study for examples, up to 25% of the patients who received buparlisib experienced severe adverse events (Campone et al., 2018).

Consequently, future evaluation of PI3K inhibition in endocrine resistant breast cancer is focusing on inhibitors with greater selectivity to improve tolerability. In particular, reduced inhibition of the β-isoform, which is believed to be a key determinant of treatment- associated toxicity, might bring some potential benefits. These α-specific, β-sparing inhibitors have been investigated in two randomised phase 3 trials (SOLAR1, SANDPIPER) (André et al., 2019; Baselga et al., 2018). Both trials focused on patients with PIK3CA mutations in ER-positive MBC patients with prior AI therapy. Whilst alpelisib demonstrated a significant benefit with the addition of fulvestrant (SOLAR1 trial), improving the median PFS from 5.7 months to 11 months (HR, 0.65, $p<0.001$), taselisib failed to demonstrated a meaningful benefit in PFS despite showing with a small improvement of the median PFS from 5.4 months to 7.4 months (HR 0.7; $p=0.0037$).

More recently, the AKT inhibitor Capivasertib has demonstrated promising activity in patients with ER-positive metastatic breast cancer (Jones et al., 2020). In the FAKTION trial, capivasertib did not just improve PFS but also suggested a possible benefit in overall survival, irrespective of PIK3CA mutations. The phase 3 development of AKT inhibitors is ongoing.

6 Short term preoperative window studies

Short term preoperative studies (WOO), are a validated clinical trial strategy to provide a rapid and cost-effective way of assessing the impact of targeted therapies alongside endocrine agents in patients with early ER-positive breast cancer (Figure 6). These studies provide access to tissue pre and post treatment for pharmacodynamic and correlative studies, providing critical insights into the mechanisms of how the investigative drug affects the tumour environment and the detection of important biomarkers that would aid in the selection of the optimal patient population.

These trials use the 2-4-week gap between the initial diagnostic biopsy and definitive surgery to treat patients with endocrine therapy +/- additional new targeted treatments to directly assess the treatment effect on the cancer. As 2-4 weeks are generally too short to see a significant change in the size of the tumour, these trials rely on changes in tumour characteristics before and after treatment, with the nuclear proliferation marker Ki67 generally being used as an intermediate endpoint of treatment benefit (Dowsett et al., 2007, 2005; Ellis et al., 2008; Hadad et al., 2015; Macaskill et al., 2011; Polychronis et al., 2005). Multiple trials have shown that reduced Ki67 expression after two weeks of preoperative endocrine therapy is linked with response after 3-4 months of treatment and, more importantly, with improved recurrence-free survival (RFS) in ER positive breast cancer patients (Dowsett et al., 2007).

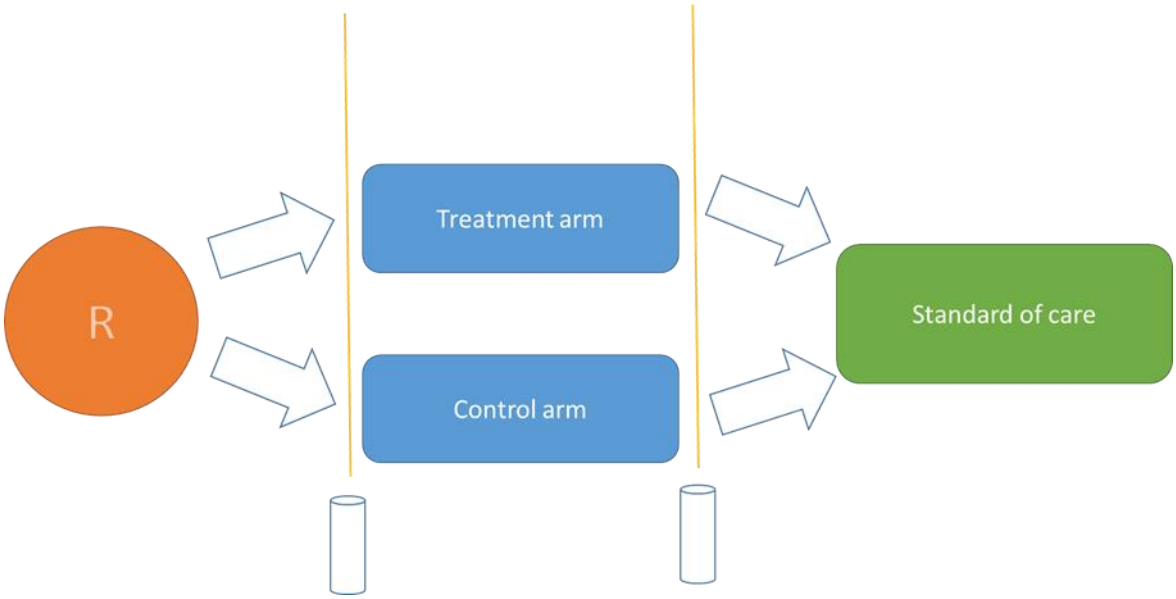


Figure 6 A simplified view of a window-of-opportunity trial. Patients recruited to the trial are randomised into treatment or control arms and samples to assess effectiveness of treatment are collected prior and post administration of trial drug. Assessment of efficacy is carried out using surrogate markers. At the end, all patients receive standard of care.

Access to tumour tissue before and after treatment also enables comprehensive analysis of biomarker changes, thus providing critical insights into the optimal patient population, biomarker responses and potential mechanisms of resistance. Although Ki67 measurements in preoperative window studies cannot replace the need for adjuvant trials with clinical endpoints, they can greatly aid in the selection or rejection of candidate approaches for phase III studies and in defining the most appropriate patient populations. Over recent years, the perioperative window setting of these studies together with the incorporation of primary biological endpoints has been established as a novel approach for breast cancer research. There is further testing of this hypothesis of improved molecular prognostication when conducted on tumours after short term endocrine treatment in the Peri-operative Endocrine Treatment for Individualising Care (POETIC) trial (Robertson et

al., 2018). If in this trial expression of ki67 or other molecular markers are found to have clinically significant value for predicting recurrence – free survival, this would greatly impact future work requiring only minimal resources to deliver this type of predictive testing routinely (Dowsett et al., 2007).

7 Rationale for the Opportune study

Pictilisib is a novel, selective, small-molecule inhibitor of Class I PI3K being developed by Genentech as an anti-cancer therapeutic. It is a potent inhibitor of the kinase activity of recombinant human p110a/p85a, with mean IC_{50} values of 8.0 and 3.4 nM. Pictilisib is also equipotent for both H1047R and E545K p110a mutants and potently inhibits other members of the Class I PI3K family (p110b/p85a, p110d/p85a, and p110y), with IC50 values of <75 nM (scintillation proximity assay). It binds Classes II, III, and IV PI3K family members weakly, or not at all including DNA-dependent protein kinase (DNA-PK) and mTOR, suggesting that pictilisib is a pan-inhibitor of the Class I PI3K family members.

At the time the OPPORTUNE trial was established, antitumor activity of Pictilisib had been observed in multiple mouse xenograft models, including breast, and lung cancer cell models. In addition, correlative downstream PD markers of PI3K activity such as phosphorylated AKT (pAKT), phosphorylated S6 (pS6), and phosphorylated PRAS40 (pPRAS40) were suppressed at doses that were consistent with efficacy in these xenograft models

Gene expression signatures associated with PI3K activity demonstrated substantial overlap between mutant and wild type patients, suggesting other mechanisms aside

from mutational activation may drive signalling through the pathway (López-Knowles et al., 2014) and emphasising the challenges of patient stratification in a pathway characterised by multiple regulatory nodes and extensive crosstalk with other signalling networks (Miller et al., 2011). These data highlighted the need for comprehensive molecular profiling of ER-positive breast cancer in order to identify biomarkers of response to PI3K inhibitors and to characterise patients most likely to benefit from this therapy.

The preoperative window study OPPORTUNE was therefore designed to assess whether addition of Pictilisib can increase the anti-tumour effects of anastrozole in ER-positive breast cancer and to perform a comprehensive analysis of the effects of PI3K inhibition on the tumour biology in various subsets of ER-positive breast cancer.

Chapter 2 Research aims and hypotheses

1 Establish the effect of the PI3K inhibitor pictilisib on tumour cell proliferation and apoptosis in patients with ER-positive breast cancer.

Pictilisib has shown substantial preclinical activity in preclinical ER-positive models with activity against both PIK3CA mutant and wild type models. Preclinical data have demonstrated that E2 can suppress apoptosis induced by PI3K inhibition in ER-positive breast cancer, suggesting independent PI3K- and E2-dependent cell survival mechanisms. Combination of endocrine therapy and PI3K inhibitors has furthermore demonstrated synthetic lethality with substantially increased apoptosis compared to single-agent therapy. The first aim of this project was to compare changes in tumour cell proliferation (as measured by Ki67 expression) and in tumour cell apoptosis (as measured by Caspase 3 expression) in pre and post-treatment tumour samples between patients treated with endocrine therapy alone and patients treated with the PI3K inhibitor Pictilisib plus Anastrozole. Given the correlation between proliferation and apoptosis, we also assessed the effect of study treatment on the growth index, defined as Ki67 expression divided by Caspase-3 expression.

2 Evaluate the interaction between PI3K pathway activation and benefit from PI3K inhibition and the treatment effects of pictilisib in subgroups

One of the key aims of this project was to identify possible patient subgroups that derive an increased benefit from adding pictilisib to endocrine therapy. As such, a main focus was set on evaluating the interaction between PI3K pathway activation

and treatment response. Although preclinical studies have shown that PIK3CA mutations result in up-regulation of the PI3K pathway and are predictive of sensitivity to PI3K inhibitors, their clinical significance is less clear. The PI3K pathway is a complex pathway with multiple nodes influencing and modulating pathway activation. Activating PIK3CA mutations have been shown to be associated with favourable tumour characteristics and improved outcome in ER-positive breast cancer (Loi et al. 2010; Sabine et al. 2014) and are also not predictive of response to endocrine treatment or mTOR-targeted therapies in ER-positive breast cancer (Hortobagyi et al. 2016; López-Knowles et al. 2014). One of the secondary aims of this project was therefore to assess the interaction between PIK3CA mutations, as determined by NGS, and response to anastrozole or the combination therapy. Since analysis of PIK3Ca mutations might be insufficient to establish actual pathway activation, we also evaluated two gene signatures (GS), which had been correlated in vitro with PI3KCA mutations and/or response to PI3K inhibitors. As it had been hypothesized that luminal B biology could be a determinant of suboptimal response to endocrine therapy alone and potentially therefore define a subgroup that might derive an increased benefit from combination therapy with pictilisib and anastrozole, we also studied the possible impact of a number of baseline characteristics that have been linked with luminal B phenotype were explored.

3 Investigate treatment-associated changes in gene expression and protein expression and phosphorylation in the tumour and stroma

Previous data suggested that single agent PI3K inhibition up-regulates expression of ER target genes. There is also substantial evidence that ER signalling and PI3K signalling can regulate the tumour microenvironment. We therefore investigated the treatment effects on ER target genes and on key proteins and genes involved in the PI3K pathway and cell cycle control using gene expression and RPPA analysis. Furthermore, we studied the impact of anastrozole and the combination therapy on the tumour microenvironment and immune system using gene expression signatures.

Chapter 3 Methods

1 Trial design

OPPORTUNE is an open label, randomised phase 2 window trial, comparing 2- weeks of pre-operative treatment with anastrozole or anastrozole plus pictilisib (with 2:1 randomisation favouring the combination) in untreated, postmenopausal patients with ER- positive, HER2-negative breast cancer (Figure 7).

The study had the following primary aims:

- Determine whether adding a PI3K-inhibitor to pre-operative endocrine treatment of ER-positive breast cancer patients increases the effects on tumour cell proliferation or apoptosis
- Identify predictors of sensitivity to PI3K-inhibition in order to characterize the patient population that benefits most from treatment with PI3K inhibitors, and
- Study the effects of combined endocrine and PI3K-inhibitor therapy on breast cancer biology

Treatment was given for 15 days (+/-2 days) unless there was evidence of unacceptable toxicity or if the patient requested to be released. The effects of the study treatment were assessed on tumour tissue specimens taken at baseline and after 15 days (+/-2 days) of the study treatment. The biopsies after 15 days of treatment were taken during definitive surgery provided this was performed on day 15 (+/- 2 days). Patients who did not have definitive surgery scheduled for day 15 (+/- 2 days) were required to undergo a core biopsy on day 15 to assess the effect of the

study treatment and were advised to continue treatment with anastrozole until surgery. Patients in the anastrozole plus pictilisib arm received the last dose of pictilisib within 2-4 hours prior to surgery.

Definitive surgery should be performed within 15 days from the start of the study treatment. Study treatment should be continued until surgery or biopsy. To gain further insight in the activity of the pictilisib, changes in relevant biomarkers were assessed on tumour tissue specimen obtained during definitive surgery. In addition, the study assessed the relationship between the anticipated anti-tumour activity of the study treatment and biological characteristics of subjects' tumour at baseline or at surgery.

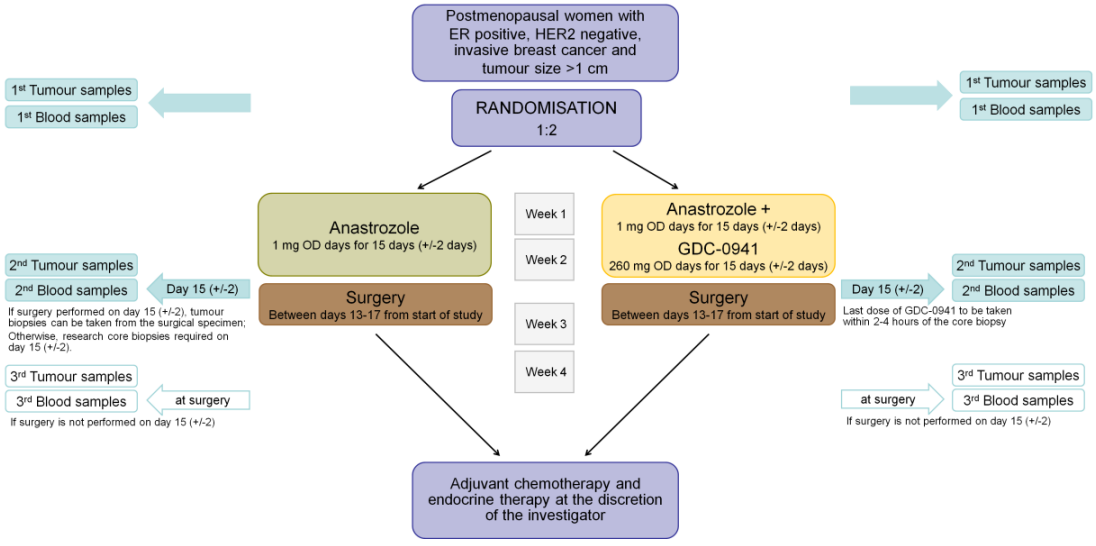


Figure 7: OPPORTUNE trial design. If patients meet the inclusion criteria, they will be randomised favourably to the combination arm. Patients participating in the trial consent for additional core biopsies at diagnosis and at 2 weeks post treatment (during surgical resection).

Patient were enrolled at 10 centres in the UK. The trial was approved by the UK MHRA and the London City East Research Ethics Committee (11/LO/1559). The trial was manged by the coordinating trial office at Brighton and Sussex Medical school (BSMS). Patient data were collected through electronic case report forms (CRFs). All patient data were verified by the trial monitor from BSMS. Clinical data for this analysis were extracted from the database after the last patient had completed their treatment and data had been verified by the clinical monitor. All patients provided written informed consent. This trial is registered under ISRCTN26131497.

Patients were eligible if postmenopausal (aged ≥55 years with amenorrhea for ≥one year, or aged <55 years with amenorrhea for ≥one year with oestradiol <20pg/mL, or prior bilateral oophorectomy) and had histologically diagnosed ER-positive, HER2-negative, invasive breast cancer. ER positive was defined as ≥1% of tumour cells positive on immunohistochemistry or an immunohistochemistry score (Allred) of ≥3. All patients had operable breast cancer ≥1 cm in diameter, adequate haematological, hepatic, and renal function, a baseline fasting plasma glucose level of <7·8mmol/L and a WHO performance status of 0–2. Prior treatment for breast cancer or use of hormone replacement therapy was not permitted. Patients with inflammatory cancer or distant metastases were excluded. Additionally, patients with significant pulmonary dysfunction, cardiac disease or diabetes mellitus were excluded.

Patients were randomly assigned (2:1, favouring the combination) to receive treatment with anastrozole or anastrozole plus pictilisib. Computer-generated permuted blocks were used, and stratification was by centre and histological grade,

as assessed on the diagnostic core biopsy. Participants and investigators were aware of assignment but the investigators who measured the biomarkers were blinded.

Anastrozole was given at a dose of 1mg OD. Pictilisib was initially administered at 340mg OD; from August 2012 onwards, the dose of pictilisib was reduced to 260mg OD following safety data from other studies indicating a lower rate of mucosal and skin toxicity at 260mg. Five evaluable patients received 340mg pictilisib; the remaining patients received 260mg. Study treatment was given for 14 days, followed by surgical resection, adjuvant chemotherapy, endocrine therapy, and radiotherapy as appropriate for each patient according to local practice guidelines.

Patients were monitored for adverse events (AEs) and changes in laboratory values, electrocardiogram, and physical examination findings.

The sample size was based on the two primary aims. A first analysis was planned for 70 evaluable patients providing 80% power at the 5% significance level (one-sided) to detect an effect size of 0.77 between ANA and ANA+PIC. Effect size was defined as $[M_{ANA+PIC} - M_{ANA}]/\sigma_{pooled}$, where $M_{ANA+PIC}$ and M_{ANA} are geometric mean Ki67 suppression values and $\sigma_{pooled} = \sqrt{[(\sigma_{ANA+PIC}^2 + \sigma_{ANA}^2)/2]}$. The study was also planned to detect a 20% difference in $R_{Ki67\text{-}Day15}$ and $R_{\Delta Ki67}$ response rates between arms. The proportion of responders in the combination group was assumed to be 60% under the null hypothesis and 80% under the alternative hypothesis; the test statistic used is the one-sided Z test with pooled variance, giving a power of 86%.

The number of samples evaluable for each endpoint was dependent on the availability of sufficient material. The protocol specified the prioritisation for tissue analyses. The primary endpoint analysis for Ki67 was prioritised; a total of 136

patients were evaluable for this analysis. For the secondary endpoint Caspase-3, 108 patients had evaluable sections pre and post treatment. Central PR and PTEN IHC review was successful in 132 and 115 patients, respectively. DNA for next generation sequencing analysis was available from the post-treatment tissue sample in 129 patients. A total of 53 and 32 paired samples were available for NanoString and RPPA analysis, respectively.

2 Tumour samples

A minimum of two core-cut tumour biopsies (14-gauge) were taken at baseline and at the end of treatment. The last dose of study medication was required within 2-4 hours before the end-of-treatment biopsy.

Biopsies for histology were placed into 10% buffered formalin within 10 minutes of sampling and fixed for ≥6 hours before processing and embedding in paraffin wax. Snap frozen cores were placed in liquid nitrogen within 10 minutes.

All tumour core biopsies were reviewed centrally at Guys Hospital London and scanned in; histological sections were assessed by H&E staining to facilitate macro-dissection of carcinoma tissue for additional biomarker analyses. Slides were subsequently reviewed independently by Prof Sarah Pinder and Dr Louise Lim.

3 Immunohistochemistry

Immunohistochemistry (IHC) for Ki67, Caspase-3, PR and PTEN was performed and analysed centrally. IHC was performed on 3-4µm sections from the FFPE core

biopsies after heat mediated antigen retrieval. Antibodies for Ki67 [Clone 30-9, Ventana], cleaved Caspase-3 [Clone Asp175, Cell Signalling], and PTEN (Clone 138G6, Cell Signalling) were used. Sections were only scored for Ki67, Caspase-3 and PTEN if the initial H&E stained section showed invasive cancer with clearly identifiable malignant epithelial cells and/or invasive tumour. For the trial, Ki67 and Caspase-3 IHC were recorded independently by two investigators, who were blinded as to treatment allocation and each other's assessment (Prof Sarah Pinder, Louise Lim).

Ki67 analysis: A minimum of 1,000 invasive cancer cells were counted for Ki67 analysis; Ki67 was scored as the percentage of positively stained cells. A cut-off of 14% was selected to define high and low baseline Ki67 expression (Macaskill et al., 2011; Yerushalmi et al., 2010). Primary Ki67 analysis was based on estimating the mean Ki67 suppression in each group and the geometric mean ratio of proportional changes between groups. Secondary Ki67 analyses were geometric mean end-of-treatment Ki67 expression, individual end-of-treatment anti-proliferative response ($R_{Ki67\text{-}Day15}$) defined as $Ln(Ki67_{Day15}) \leq 2$, and individual anti-proliferative response ($R_{\Delta Ki67}$) defined as a ≥50% fall in Ki67 expression (Maisonneuve et al., 2014; O'Brien et al., 2010).

Caspase-3 analysis: For Caspase-3, at least 3,000 invasive cancer cells were assessed, if available. Caspase-3 analyses included geometric mean change in Caspase-3 between day 15 and baseline and individual apoptotic response ($R_{\Delta Casp3}$), defined as a ≥50% increase in Caspase-3 IHC.

Progesterone receptor analysis: PR was assessed centrally and regarded as positive if Allred score was ≥3.

PTEN analysis: PTEN was classified as “positive” if any cytoplasmic and/or nuclear expression immunoreaction was observed in tumour cells and “negative” if no immune reactivity was observed, with the surrounding tissue microenvironment serving as a positive internal control.

4 DNA/RNA extraction

Following macro-dissection for tumour-enriched areas with >70% malignant tissue, DNA and RNA were simultaneously extracted from FFPE sections using Qiagen AllPrep DNA/RNA FFPE kit as per manufacturer’s instructions. FFPE sections in tube were dewaxed and rehydrated using xylene-based protocol, and DNA/RNA extracted in a column-based approach. Nucleic acids were quantified and checked for purity using a UV spectrophotometer (NanoDrop).

5 Gene expression analysis

RNA expression analysis of approximately 800 breast cancer-related genes using the nCounter platform (NanoString Technologies, Seattle, US). The samples from this study were serial sections from FFPE tissue used for Ki67 determination. RNA analysis was performed at Genentech, Inc., South San Francisco, CA, US. RNA (100ng) was hybridized overnight at 65°C according to the NanoString protocol. Samples were subsequently loaded onto the NanoString nCounter Prep Station and transcripts were counted using the NanoString nCounter Digital Analyzer at a FOV of 280.

Samples were normalized to housekeeping genes. PAM50 analysis of Luminal A and Luminal B subtypes was carried out as previously reported (Parker et al., 2009). Data were transferred back for integrated analysis.

6 Next Generation Sequencing

Analysis of mutations and copy number changes of PIK3CA and other key pathway components was assessed by targeted next generation sequencing using the Ampliseq Comprehensive Cancer panel assay with the Ampliseq Library Kit 2.0 according to the manufacturer's instructions (ion torrent, life technologies, US). NGS analysis was performed at the Centre for Personalized Nanomedicine at the Australian Institute for Bioengineering and Nanotechnology, University of Queensland, Australia. NGS analysis was supported by a grant from the National Breast Cancer Foundation (NBCF) of Australia (CG-12-07).

Samples underwent 19 rounds of amplification and were barcoded using the Ion Xpress barcodes (Ion torrent, LifeTechnologies). Each pool was quantified post-adaptor ligation by qPCR. Samples were pooled to provide 300x coverage. The Ion PI Template OT2 200 v3 Kit, P1 chip and Ion PI Sequencing 200 v3 Kit were used as per the manufacturers protocol. Variant calling used the Torrent variant Caller (v4.0-r76860) set on Somatic PGM low stringency settings. Torrent Suite 4.0.2 was used for data processing, base-calling, and mapping. Data were transferred back for integrated analysis.

7 Reverse Phase Protein Arrays

Reverse Phase Protein Arrays (RPPA) analysis of 55 targets focused on PI3K pathway signalling, ER signalling, alternative intracellular signalling and cell cycle regulation. RPPA analysis was performed at Theranostics Health, Inc., Gaithersburg, MD, USA. 32 matched pairs of patient flash frozen samples were selected for this study. Whole slide lysates were prepared and approximately 6nl of protein were printed in 4 replicates of glass backed nitrocellulose slides. Protein was printed at approximately 2 concentrations, 0.5mg/ml or 0.25mg/ml. Slides were incubated with 55 different antibodies (see Appendix) and target specific signal was captured at 635nm. The amount of protein printed at each spot was measured using a Sypro Ruby Protein Blot Stain (Invitrogen: S11791), captured at 532nm. The total protein yield is used as a denominator for primary antibody signal, giving us a total protein normalised signal. All results presented use the total protein normalised signal fit on a LOESS algorithm. Data were transferred back for integrated analysis.

8 Statistical Analysis

The main study endpoints are listed in Table 2:

Primary Objective	**Endpoints**
Determine the activity of the study treatment on tumour-cell proliferation	• The difference in geometric mean change (post-pre) in Ki67 expression between two treatment groups • To explore further, this will also be measured in patients with and without PI3K mutations and/or loss of PTEN

Secondary objectives	**Endpoints**
Determine the activity of the study treatment on tumour-cell proliferation	Secondary Ki67 analyses: • Geometric mean Ki67 expression at the end of study treatment (Mean $Ki67_{post}$). • Individual end-of treatment anti-proliferative response ($Response_{Ki67\text{-}Post}$), defined as the natural logarithm of percentage Ki67 positive cells of less than 1 or 1-2 at the end of study treatment • Individual anti-proliferative response ($Response_{\Delta Ki67}$), defined as a ≥50% fall in Ki67 expression over the course of the study treatment
Determine the effects of the study treatment on tumour-cell apoptosis	Changes in the Caspase3 assay between pre- and post-treatment tumour samples: • Geometric mean change in Caspase3 assay between end-of-treatment and pre-treatment tumour samples (Mean Δ Caspase3). • Individual apoptotic response ($Response_{\Delta CASPASE3}$), defined as a ≥50% increase in Caspase3 assay over the course of the study treatment • Individual end-of treatment apoptotic response ($Response_{Caspase3\text{-}Post}$), defined as the natural logarithm of percentage Caspase3 positive cells of less than 1 or 1-2 at the end of study treatment
Tertiary/Exploratory objectives	**Endpoints**
Evaluate potential biomarkers that may help predict response to anastrozole and/or pictilisib	Alterations in DNA and RNA, including mutational status, RNA expression levels, DNA copy number, and protein expression
Explore the biologic effects of anastrozole and/or pictilisib on breast cancer and stromal cells and establish pharmacodynamic markers of anastrozole and/or pictilisib action	
Explore mechanisms of resistance	

Table 2 Study Objectives and Endpoints

Populations for analysis: All analyses for Ki67 and Caspase3 changes were performed on a per- protocol population, defined as all patients who completed two weeks of treatment and for whom tumour biopsy specimens were available for assessment of biological response. Patients excluded from the Per-Protocol-Population were replaced.

Sample Size: The planned study size was 94 evaluable patients in the anastrozole plus pictilisib group and 47 evaluable patients in the anastrozole group, respectively, to provide 80% power to detect an effect size of 0.58 between Anastrozole and Anastrozole + Pictilisib at the 5% significance level. The effect size (ES) was defined as the treatment difference divided by the standard deviation, i.e. ES = $[M_1 - M_2]/ \sigma$ pooled, where M1 and M2 are the mean values of the differences of proportional Ki67 changes and σpooled = $\sigma [(\sigma_1^2 + \sigma_2^2)/2]$. Taking Cohen´s standard interpretation of effect sizes into account, 0.5 is the lower limit of medium effect. An effect size of 0.5 corresponds to 33% of non-overlap between the two treatment groups. The non-centrality parameter δ is 2.83. Critical t is 1.98.

Group sample sizes of 47 patients in the Anastrozole group and 94 in the Anastrozole + pictilisib group also achieved 80% power to detect a difference between the group response rates of 20%. Response was defined as a 50% or higher fall in Ki67 expression. The proportion of responders in the Anastrozole + pictilisib group was assumed to be 60% under the null hypothesis and 80% under the alternative hypothesis. The proportion in the Anastrozole group was assumed to be 60%. The test statistic used was the one-sided Z test with pooled variance. The significance level was 5.1%. If the difference between the group response rates was 25%, then

the sample size needed was 28+56=84. On the other hand, if the difference was 30%, then only 18+36=54 patients were sufficient to detect this difference.

Individual end-of treatment anti-proliferative response (ResponseKi67-Post), defined as the natural logarithm of percentage Ki67 positive cells of less than 1 or 1-2 at the end of study treatment, was another endpoint under which required sample size might be slightly lower.

Assuming that approximately 40% of patients have activating PI3KCA mutations or PTEN deletions, the study had 80% power at a 5% significance level to detect an ES of 0.8.

Efficacy analysis: The main analysis of apoptosis and proliferation were from baseline to day 15 using non-parametric statistics to compare the log (surgical/Pre-treatment) scores. Additional analyses of apoptosis and proliferation were from day 15 to definitive surgery.

Treatment comparisons were tested with and without adjustment for baseline prognostic factors. In the absence of major confounding factors, the latter were considered secondary endpoints.

On the assumption of a log normal distribution, Ki67 values were log transformed before analysis of mean Δ_{Ki67}, mean Ki67post, and $Response_{Ki67}$-Post. If Ki67 is not normally distributed, nonparametric, distribution-free tests like Wilcoxon Signed-Rank Test, Sign Test or Wilcoxon Mann-Whitney test can be applied. Ln(Ki67post) and ln(Ki67pre) were used to calculate the geometric means. 0.1 was be added to every untransformed Ki67 value to avoid the mathematical anomaly that arises because

the log of zero is minus infinity. As a consequence of the assumption of a lognormal distribution, ln(Ki67post)–ln(Ki67pre) was also normally distributed. This formula gives the proportional change, and as a result mean log proportional changes and CI can be calculated and displayed on their original scale by back transformation. Mean ΔKi67 and mean Ki67post were compared between groups by use of the t- test, and the proportional change within groups was analysed with the paired t- test. The proportional reduction was calculated as one minus the proportional change.

Anti-proliferative response ResponseΔKi67 and end-of treatment anti-proliferative response ResponseKi67-Post were calculated in all evaluable patients. An estimate of the anti-proliferative response rates RRΔKi67 and end-of treatment anti-proliferative response rates RRKi67-Post and 95% CIs (Clopper and Pearson, 1934) was calculated for each treatment arm. CIs for the difference in response rates (Berger and Boos, 1994; Santner and Snell, 1980) were calculated. The relative risk (treatment:control) was reported along with the associated 95% confidence interval based on logistic regression model.

A similar analyses strategy was applied for Caspase3 endpoints.

Changes in secondary outcomes from baseline to post treatment were analysed between treatment groups with the Wilcoxon Mann-Whitney test and within treatment groups with the Wilcoxon signed rank test. Associations between outcomes were investigated by use of the Spearman's rank correlation coefficient. A comprehensive statistical-analysis plan was prepared before un-blinding of the data.

Subgroup analysis: The effects of the study treatment were assessed separately in patients with and without PI3K mutations and/or PTEN deletions, Luminal A and B

subtypes and patients with high (>14%) or low (≤14% baseline Ki67). Additional subgroups were defined by the exploratory biomarker analysis.

Statistical analyses for the trial were performed by Dr Shah-Jalal Sharker, trial statistician at the Centre of Experimental Cancer Medicine at Queen Mary University London.

9 Summary of individual contribution

Dr Lim oversaw the collection and cleaning of the clinical data as well as the collection and processing of the tissue samples. Following staining and scanning of the FFPE sections at Prof Pinder's laboratory, Dr Lim reviewed and scored all samples for Ki67 and Caspase-3 expression. The results were documented in the trial data base together with the independent scoring by Prof Pinder. Dr Lim was responsible for macro-dissection of tissue samples and subsequent DNA and RNA extraction and quantification as outlined in the methods section. She coordinated analysis of RNA and DNA samples at NanoString Technologies laboratories (Seattle, US), Theranostics Health (Gaithersburg, MD, USA) and the University of Queensland (Brisbane, Australia), respectively. Dr Lim coordinated transfer and integration of the translational data sets and the clinical data sets and assisted Dr Shah-Jalal Sharker with the statistical analysis and interpretation of the data.

Chapter 4 Results

1 Effect of Study Treatment on Tumour Cell Proliferation

Between January 2012, and September 2015, 167 patients underwent randomization (Figure 8). 54 patients were assigned to anastrozole alone and 113 patients to anastrozole plus pictilisib. Two patients were excluded because of violations of key eligibility criteria. Another two patients withdrew trial consent prior to the start of their study treatment. Assessment of the treatment effects was possible for 136 patients who successfully completed the protocol; 27 patients (8 in the anastrozole arm and 19 in the combination arm) had insufficient tissue for analysis.

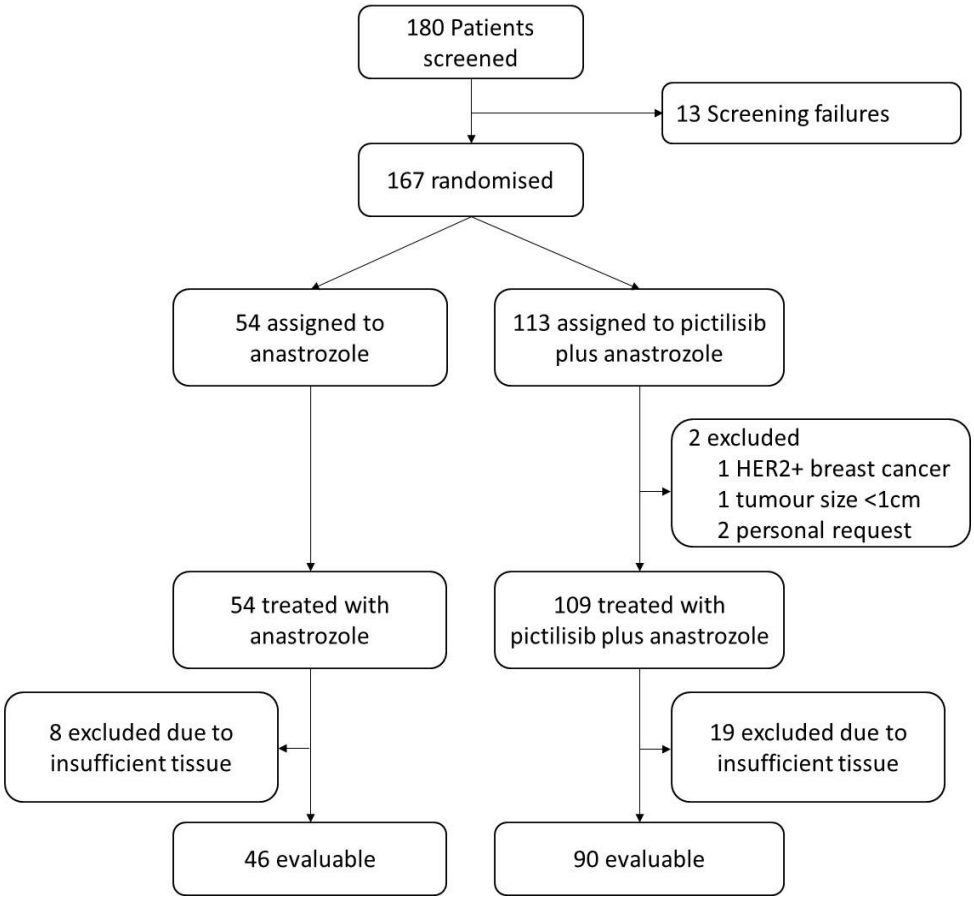


Figure 8: Trial consort diagram

Baseline distributions of patient and tumour characteristics were similar in the treatment arms (Table 3); 62% of tumours were classified as Luminal B according to PAM50 analysis and 63.2% according to baseline Ki67 analysis using a cut-off of 14%. 58.8% of tumours were PIK3CA wildtype. There was a slightly higher number of patients with PR positive tumours in the combination group.

	Anastrozole alone (n = 46)	**Pictilisib plus Anastrozole (n = 90)**
Age (years)		
Median (range)	66.9 (47.7-85.4)	64.1 (48.5-81.1)
Tumour status		
Grade 1	5 (10.9%)	13 (14.6%)
Grade 2	34 (73.9%)	62 (69.7%)
Grade 3	7 (15.2%)	14 (16.9%)
PR status		
Positive	33 (71.4%)	82 (91.1%)
Negative	11 (23.9%)	6 (6.7%)
Molecular Subtype (PAM50)		
Luminal A	6 (31.6%)	14 (41.2%)
Luminal B	13 (68.4%)	20 (58.8%)
Ki67 (% positive tumour cells)		
Mean (range)	23.0 (1.9-84.1)	22.7 (0.9-89.9)
0-14	15 (32.6%)	35 (38.9%)
>14	31 (67.4%)	55 (61.1%)
PIK3CA mutation status *		
Wildtype	27 (58.7%)	53 (58.9%)
Mutation	19 (41.3%)	30 (33.3%)
Kinase-domain mutation	14 (30.4%)	15 (16.7%)
Helical-domain mutation	5 (10.9%)	14 (15.6%)

*Table 3 Patient demographics and tumour characteristics at baseline; Kinase-domain mutations include H1047R/Y, H1048R, G1049D/R; Helical-domain mutations include E524K, E545K; * All patients in the anastrozole arm were evaluable for PIK3CA mutations analysis; 7 patients in the Pictilisib and anastrozole arm were not available for PIK3CA NGS analysis; the percentage value refers to all patients in the pictilisib and anastrozole*

Figure 9 demonstrates the individual Ki67 results for the two separate assessments. There was high concordance between both readers. The mean (median) difference between the 2 analyses was 3.90% (3.06%) for the baseline assessment and 2.63% (1.72%) for the end of treatment analysis, respectively. When using a 14% cutoff for Ki-67, the Cohen's kappa value (Cohen, 1960) at baseline was 0.77 and at end of treatment is 0.77, indicating a substantial agreement between the independent scorers.

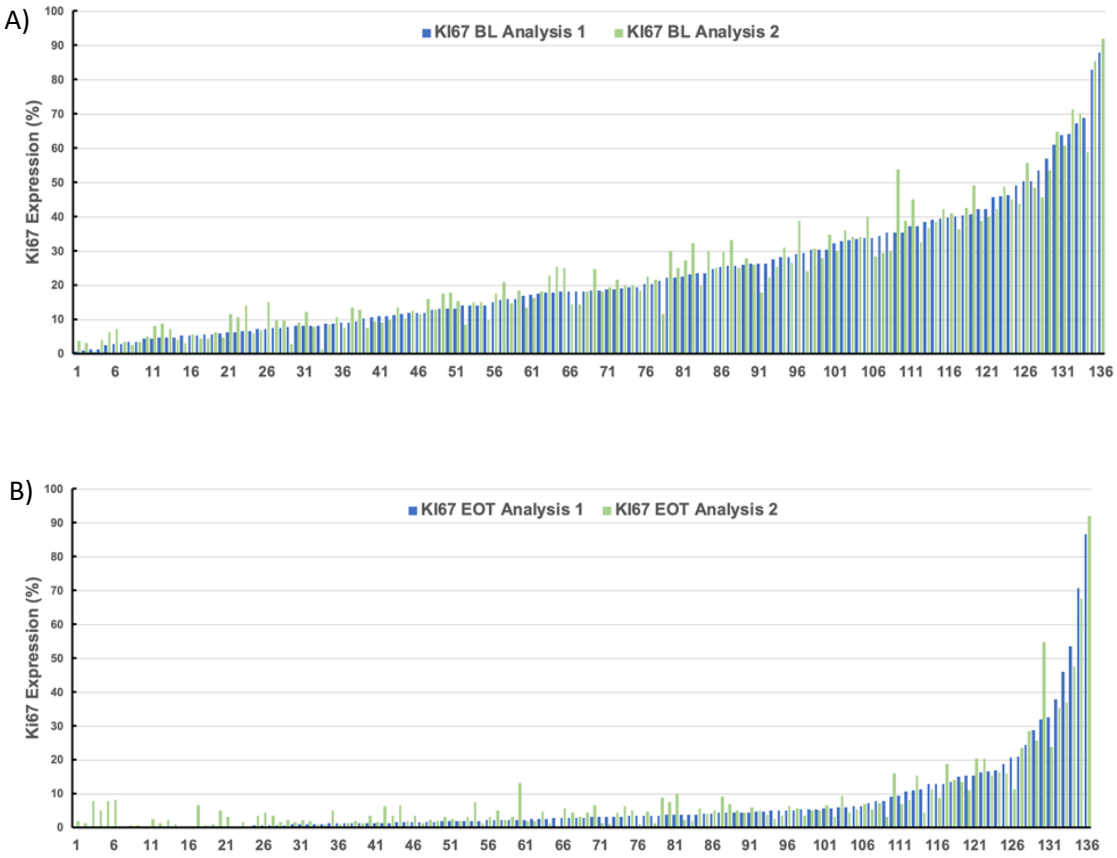
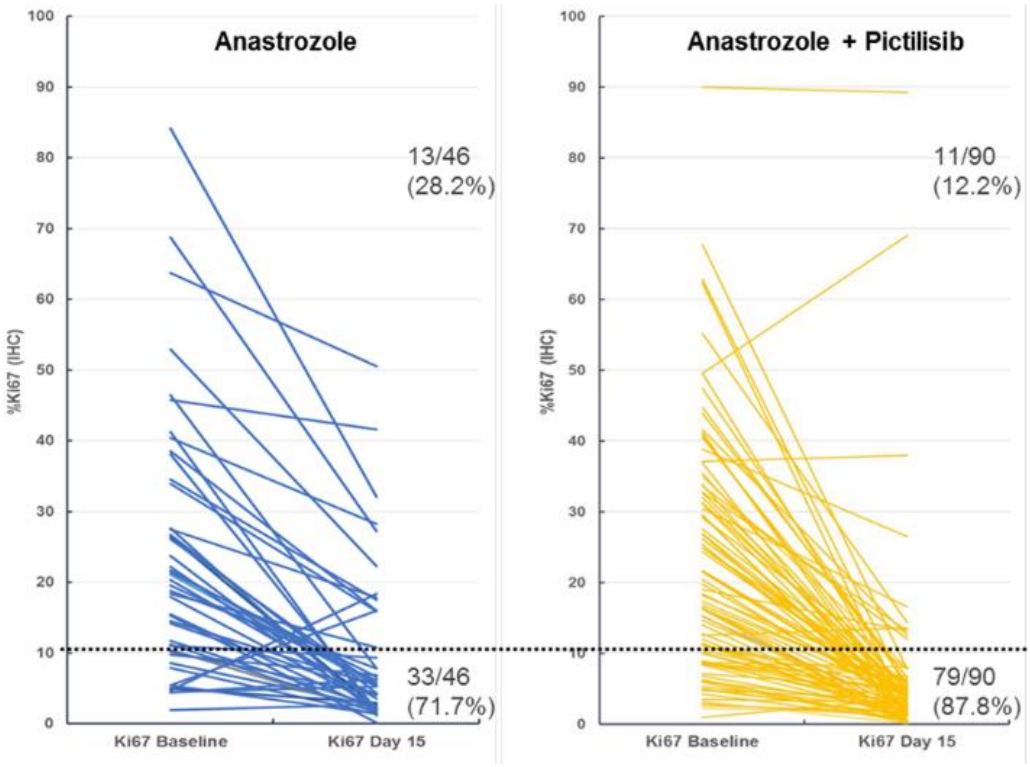


Figure 9: Comparison of the two independent Ki67 analyses at baseline (a) and at EOT (b)

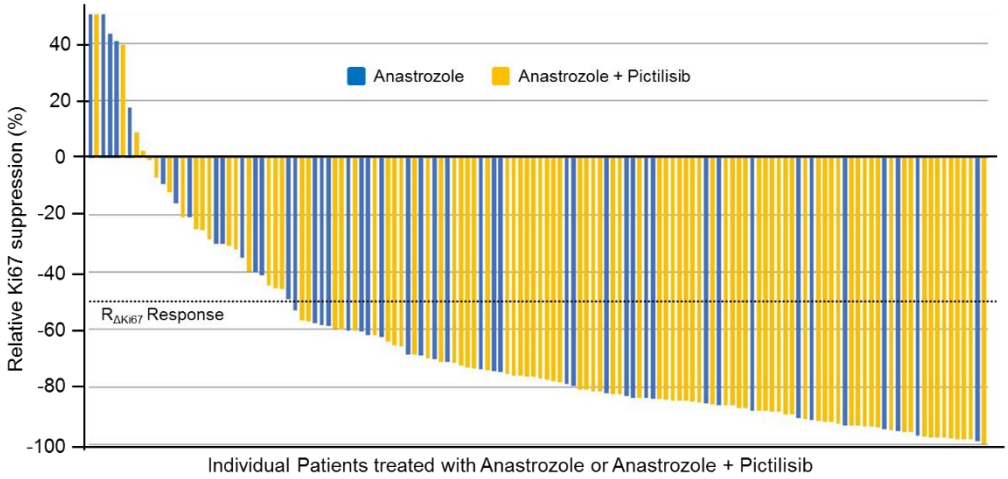
Tumour Ki67 expression decreased in the 93.4% of patients over the course of the study treatment from baseline to day 15 (Figure 10a, b); in 9 patients Ki67 expression numerically increased, including 5 patients in the anastrozole group (10.9 of all patients treated with anastrozole) and 4 patients in the combination group (4.4%). In 4 of the patients with a numerical increase in Ki67 expression, the pre-treatment and EOT Ki67 remained under 10% and absolute changes in expression were low. More patients in the combination group (87.8%) had an EOT Ki67 expression of <10% compared to anastrozole alone (71.7%).

Mean percentage suppression of Ki67 was 82.5% (95% CI, 78.3%-85.8%) for anastrozole plus pictilisib treated patients and 70.7% (61.0%-78.0%) for anastrozole treated patients (Table 4; Figure 10 A). The ratio (combination/anastrozole) of mean Ki67 suppression was 0.60 (0.58-0.85; p=0·01). The geometric mean end of treatment Ki67 expression was 6.3% (3.7%-8.8%) for anastrozole plus pictilisib and 9.5% (6.3%-12.8%) for anastrozole alone (p=0.02). The EOT response rate RKi67-Day15 was higher with the combination 83.3% (76.8%-90.9%) compared to anastrozole alone 65.2% (53.3%-77.1%; p=0.02).

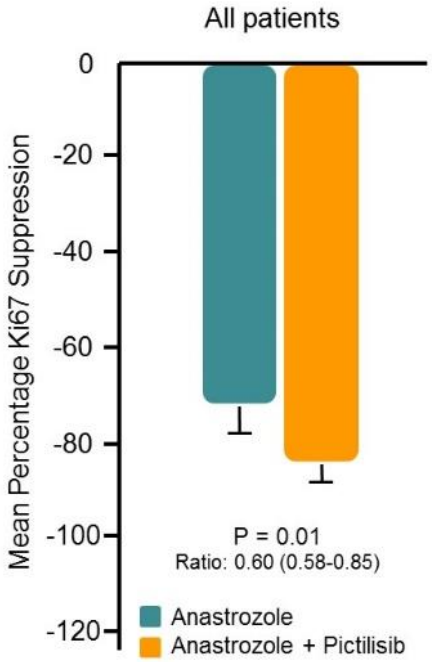
A)


Anastrozole
Anastrozole + Pictilisib
%Ki67 (IHC)
13/46
(28.2%)
33/46
(71.7%)
11/90
(12.2%)
79/90
(87.8%)
Ki67 Baseline
Ki67 Day 15

B)


Anastrozole
Anastrozole + Pictilisib
Relative Ki67 suppression (%)
R_ΔKi67 Response
Individual Patients treated with Anastrozole or Anastrozole + Pictilisib

C)



D)

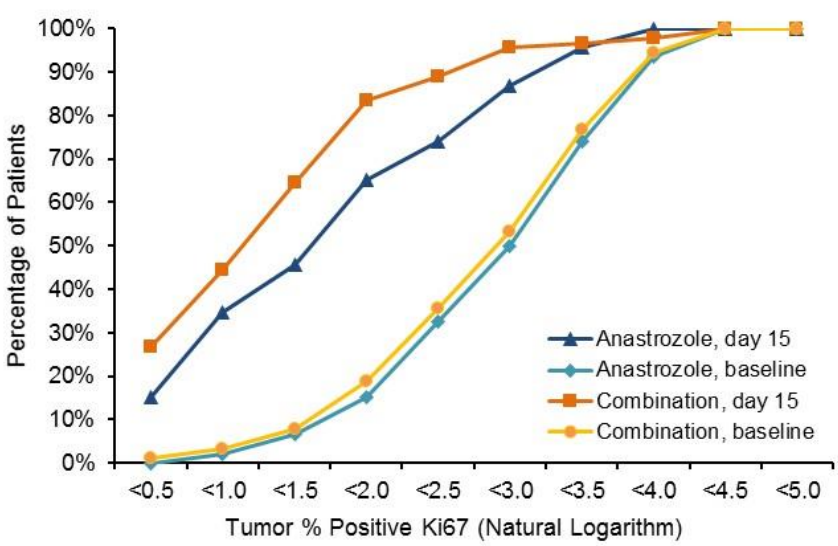


Figure 10: Individual Ki67 changes from baseline to Day 15 and Anti-proliferative response to study treatment; a) Individual changes in percentage Ki67 expression from baseline to Day 15; the number and percentage of patients achieving an end of treatment (EOT) Ki67 score of >10% or ≤10% are provided for each group; b) Individual relative Ki67 suppression sorted from low to high; relative Ki67 Suppression is defined as Ln(Ki67Day15) – Ln(Ki67baseline); results are displayed on their original scale by back transformation. c) anti-proliferative response expressed as the geometric mean Ki67 suppression in from baseline to day 15; error bars indicate 95% CI; d) anti-proliferative response at day 15 compared with baseline. The cumulative proportion (by percentage) of patients who had tumours with percentage positive Ki67 (expressed as the natural logarithm) less than the value on the X axis is illustrated at baseline and at day 15 for each treatment arm

	Anastrozole (n = 46)	Pictilisib plus Anastrozole (n = 90)	Relative Risk (combination/ anastrozole)	p-Value
Geometric mean Ki67 suppression [% (95% CI)]	70.7% (61.0%-78.0%)	82.5% (78.3%-85.8%)	0.60 [1] (0.58-0.85)	p=0.01
The geometric mean EOT Ki67 expression [% (95% CI)]	9.5% (6.3%-12.8%)	6.3% (3.7%-8.8%)	0.66 [1] (0.58-0.69)	p=0.02
$R_{\Delta Ki67}$ response rate [% (95% CI)]	69.6% (58.0%-81.1%)	81.1% (74.2%-88.0%)	1.17 (0.97-1.40)	p=0.10
$R_{Ki67\text{-}Day15}$ response rate [% (95% CI)]	65.2% (53.3%-77.1%)	83.3% (76.8%-90.9%)	1.36 (1.05-1.55)	p=0·02

Table 4 Anti-proliferative response to anastrozole or anastrozole plus pictilisib; geometric mean Ki67 suppression is defined as Ln(Ki67Day15) - Ln(Ki67baseline); the ratio (combination/ anastrozole) of geometric mean Ki67 suppression is provided with 95% CI. Geometric mean end-of-treatment (EOT) Ki67 expression is defined as Ln(Ki67Day15)]; individual EOT anti-proliferative response RKi67-Day15 is defined as Ln(Ki67Day15) ≤2; individual anti-proliferative response RΔKi67 is defined as a ≥50% fall in Ki67 expression between baseline and Day 15. 1 Geometric mean ratio of Ki67 proportional changes between the groups

2 Effect of Study Treatment on Tumour Cell Apoptosis

Overall, the rate of apoptosis at baseline and end of treatment was extremely low throughout the trial limiting the ability to assess possible differences between treatment groups (Table 5).

In contrast to previous reports, we were unable to demonstrate a clear correlation between Ki67 and apoptosis (Figure 11), We analysed the growth index defined as percent Ki67-expresion divided by percent Caspase-3 expression (Table 6). There was a greater suppression in the growth index in the combination arm (75.2%) compared to anastrozole alone 55.9% (Figure 12), but was not statistically significant.

	Anastrozole (n = 33)	**Pictilisib plus Anastrozole (n = 56)**	**Relative Risk (combination/ anastrozole)**	**p-Value**
The geometric mean baseline Casp-3 expression [% (95% CI)]	0.14% (0.10%-0.18%)	0.15% (0.11%-0.19%)	NS	NS
The geometric mean EOT Casp-3 expression [% (95% CI)]	0.14% (0.11%-0.18%)	0.14% (0.10%-0.19%)	NS	NS

Table 5 Induction of apoptosis with anastrozole or anastrozole plus pictilisib; geometric mean Ki67 suppression is defined as Ln(Ki67Day15) - Ln(Ki67baseline); the ratio (combination/ anastrozole) of geometric mean Ki67 suppression is provided with 95% CI. Geometric mean end-of-treatment (EOT) Ki67 expression is defined as Ln(Ki67Day15)]; individual EOT anti-proliferative response RKi67-Day15 is defined as Ln(Ki67Day15) ≤2; individual anti-proliferative response RΔKi67 is defined as a ≥50% fall in Ki67 expression between baseline and Day 15.

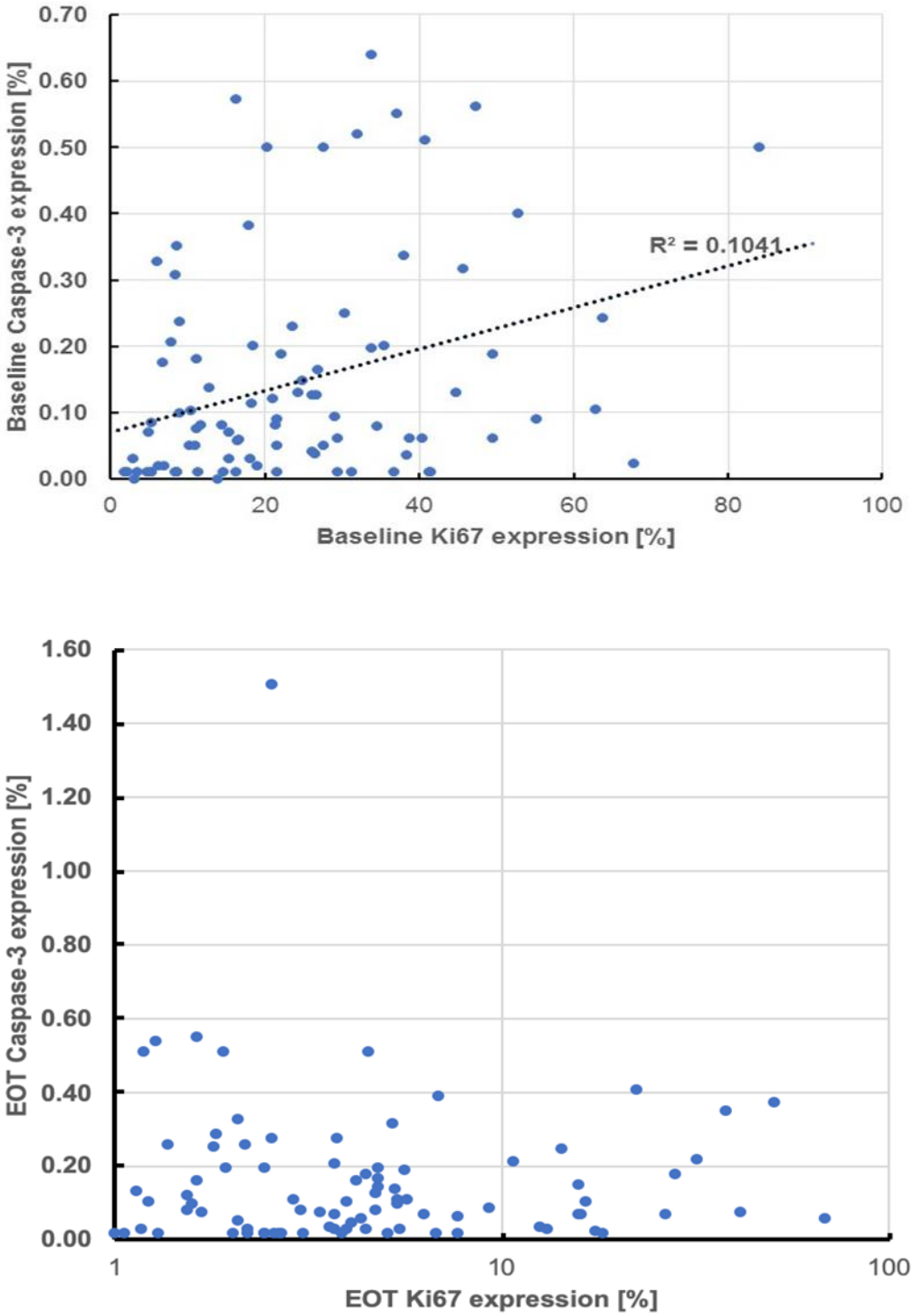


Figure 11: Relationship between Ki67(%) and apoptosis (%) before and after 2 weeks of treatment irrespective of treatment arm.

	Anastrozole (n = 33)	Pictilisib plus Anastrozole (n = 56)	Relative Risk (combination/ anastrozole)	p-Value
The geometric mean baseline growth index [% (95% CI)]	433.2 (190.0-676.4)	635.5 (381.2-889.8)	NS	NS
The geometric mean EOT growth index [% (95% CI)]	190.9 (71.5-310.3)	157.5 (93.0-222.1)	NS	NS
The mean relative growth index suppression [% (95% CI)]	55.9 (4.4-270.7)	75.2 (25.3-191.8)	NS	NS

Table 6 Treatment-associated change in growth index (GI), defined as Ki67[%]/Caspase-3[%], with anastrozole or anastrozole plus pictilisib

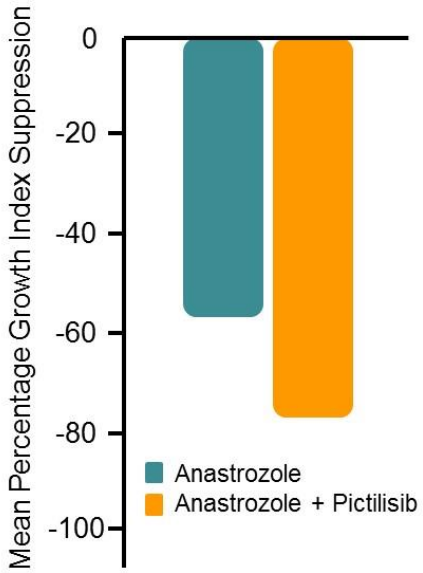


Figure 12: Mean percentage Growth Index suppression

3 Effect of study treatment in subgroups defined by PIK3CA mutations, Luminal A/B subtypes and baseline Ki67 scores

PIK3CA mutation subtypes and response to study treatment:

Three major hotspots of mutations of the PIK3CA gene have been described; these are concentrated in the helical (E542K and E545K) and kinase (H1047R) domains, accounting to approximately 90% of all PIK3CA mutations (Ellis et al., 2010). In the OPPORTUNE trial, we tested for PIK3CA mutations using NGS. At least one PIK3CA mutation was detected in 49 tumours (36.0%), including 19 helical domain and 29 kinase domain mutations. There was no significant correlation between PIK3CA mutation and added activity of pictilisib (Table 7); the ratio (combination/anastrozole) of geometric mean Ki67 proportional change was 0.63 (0.39–1.0; p=0.05) for patients with PIK3CA-wildtype tumours and 0.72 (0.46–1.15; p=0.12) for patients with PIK3CA-mutated tumours.

Interestingly, a significant interaction was observed between PIK3CA mutation subtypes [helical domain mutations (HD), kinase domain mutations (KD), wildtype (WT)] and mean Ki67 suppression (Figure 13); the combination/anastrozole geometric mean ratio of Ki67 suppression was 0.48 (0.27-0.84; p=0.02) for patients with HD mutations and 0.63 (0.39–1.0; p=0.05) for patients with PIK3Ca WT, compared to 1.17 (0.57–2.41; p=0.64) for patients with KD mutations. This was largely due to patients with HD mutations showing a particularly poor response to anastrozole alone [mean Ki67 suppression 53.9% (9.5%-76.5%)], that was reversed by the addition of pictilisib [mean Ki67 suppression 78.1% (71.0%-83.4%)].

On the other hand, patients with KD mutations responded well to anastrozole alone [mean Ki67 suppression 77.7% (57.0%-88.4%)] and showed no benefit from the addition of pictilisib [mean Ki67 suppression 73.9% (59.8%-83.0%)].

	Anastrozole (n = 46)	**Pictilisib plus Anastrozole (n = 90)**	**Relative Risk (combination/ anastrozole)**	**p-Value**
Geometric mean Ki67 suppression [% (95% CI)]				
PIK3Ca WT	69.9 (45.7 – 80.2)	81.1 (75.2 – 85.6)	0.63 (0.39–1.0)	0.05
HD mutations	53.9 (9.5 - 76.5)	78.0 (71.0 – 83.3)	0.48 (0.27-0.84)	0.02
KD mutations	77.7 (57.0-88.4)	73.9 (59.8 – 83.0)	1.17 (0.57–2.41	0.64
$R_{Ki67\text{-}Day15}$ response rate [% (95% CI)]				
PIK3Ca WT	59.3 (42.8-75.7)	84.9 (76.6-93.2)	1.43 (1.08-1.89)	0.01
HD mutations	75.0 (16.2-133.8)	84.6 (66.1-103.3)	1.13 (0.68 -1.88)	0.58
KD mutations	81.8 (59.7-103.9)	78.6 (58.4-98.7)	0.96 (0.69-1.33)	0.62

Table 7 PIK3CA status and anti-proliferative response to anastrozole or anastrozole plus pictilisib; geometric mean Ki67 suppression is defined as Ln(Ki67Day15) - Ln(Ki67baseline); the ratio (combination/ anastrozole) of geometric mean Ki67 suppression is provided with 95% CI; individual EOT anti-proliferative response RKi67-Day15 is defined as Ln(Ki67Day15) ≤2.

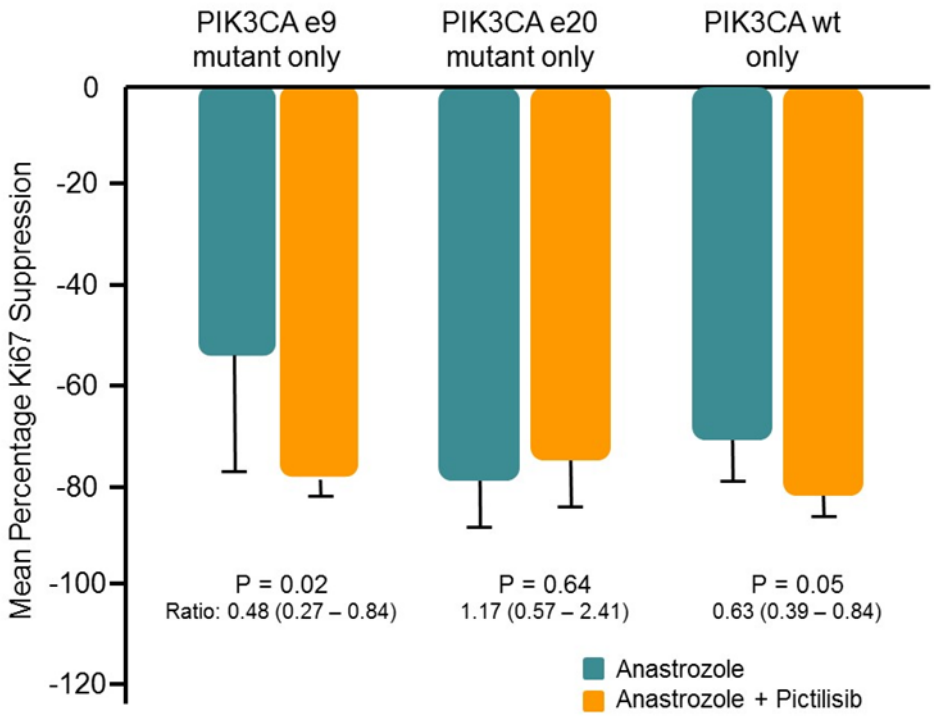
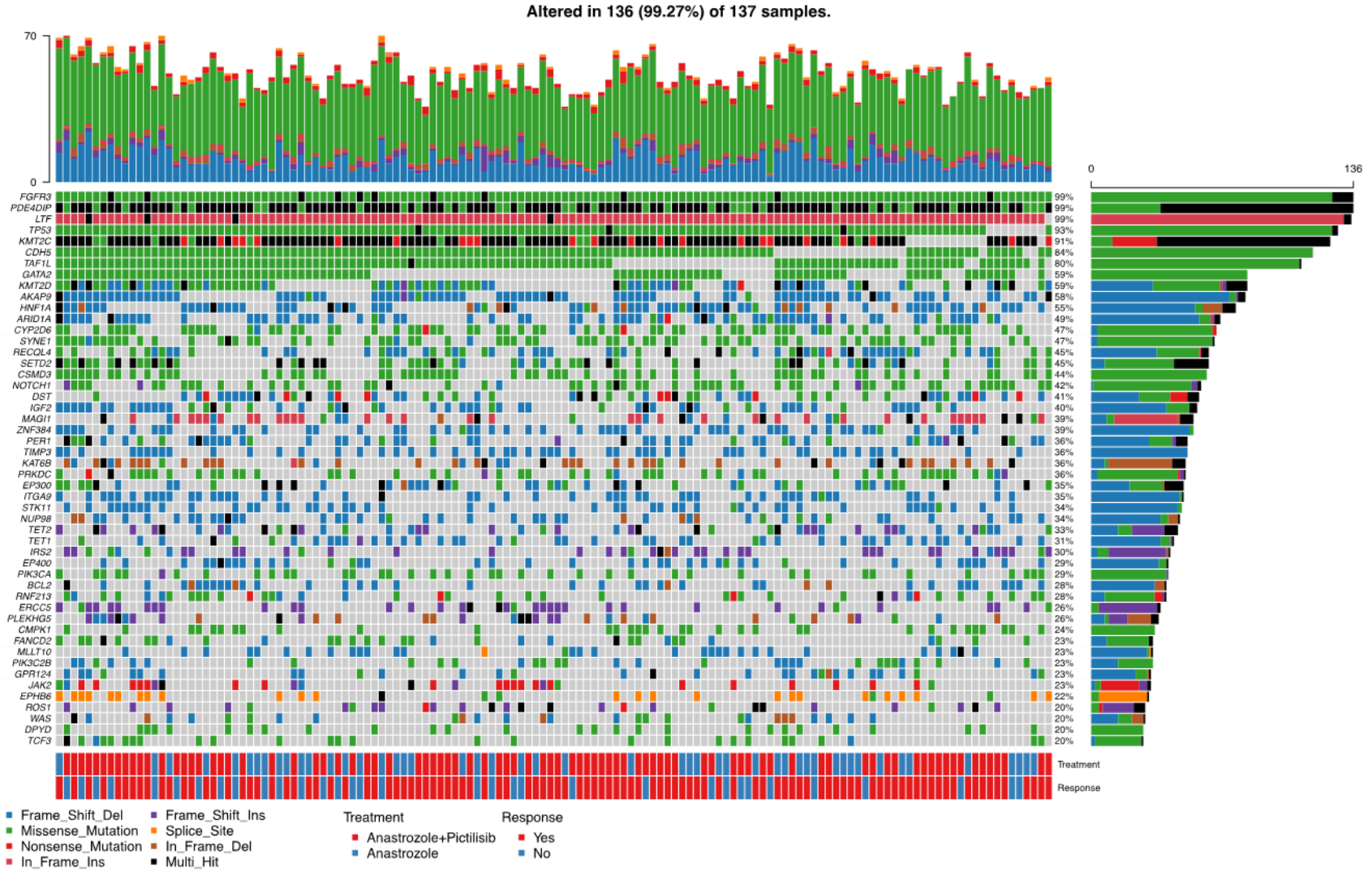


Figure 13: Anti-proliferative response to study treatment by PIK3CA mutation status; e9: exon 9 domain mutations (helical domain); e20: exon 20 domain mutations (kinase domain)

Further NGS analysis demonstrated a range of somatic mutations in keeping with the expected mutational landscape of ER-positive early breast cancer. Figure 14 shows an overview of the somatic variants for each treatment group, divided by response to treatment. There was no specific mutational pattern associated with response to anastrozole or anastrozole plus pictilisib, when looking at the number of mutations in the most frequently mutated genes using a Forest plot (Figure 14B).

A)


Altered in 136 (99.27%) of 137 samples.
70
0
0
136
FGFR3 99%
PDE4DIP 99%
LTF 99%
TP53 93%
KMT2C 91%
CDH5 84%
TAF1L 80%
GATA2 59%
KMT2D 59%
AKAP9 58%
HNF1A 55%
ARID1A 49%
CYP2D6 47%
SYNE1 47%
RECQL4 45%
SETD2 45%
CSMD3 44%
NOTCH1 42%
DST 41%
IGF2 40%
MAGI1 39%
ZNF384 39%
PER1 36%
TIMP3 36%
KAT6B 36%
PRKDC 36%
EP300 35%
ITGA9 35%
STK11 34%
NUP98 34%
TET2 33%
TET1 31%
IRS2 30%
EP400 29%
PIK3CA 29%
BCL2 28%
RNF213 28%
ERCC5 26%
PLEKHG5 26%
CMPK1 24%
FANCD2 23%
MLLT10 23%
PIK3C2B 23%
GPR124 23%
JAK2 23%
EPHB6 22%
ROS1 20%
WAS 20%
DPYD 20%
TCF3 20%
Treatment
Response
Frame_Shift_Del
Missense_Mutation
Nonsense_Mutation
In_Frame_Ins
Frame_Shift_Ins
Splice_Site
In_Frame_Del
Multi_Hit
Treatment
Anastrozole+Pictilisib
Anastrozole
Response
Yes
No

B)

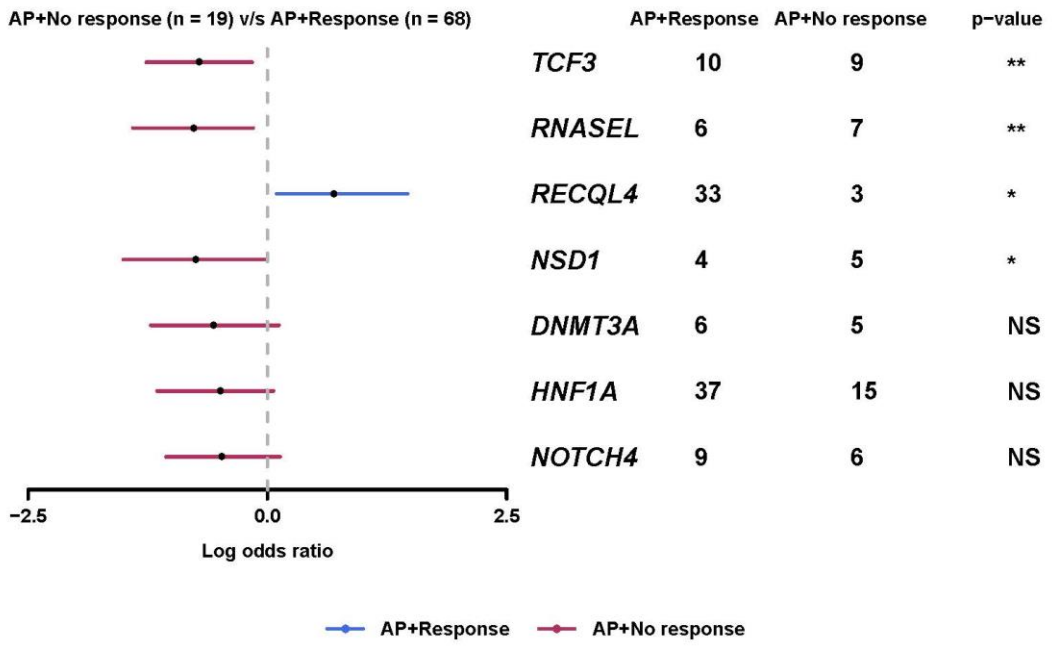


Figure 14: A) Somatic variant analysis and response to anastrozole or anastrozole/pictilisib. B) Forest plots showing the log odds ratio indicate there is no correlation between the top genes and response to treatment.

PAM50 Luminal Status and treatment response:

NanoString PAM50 analysis was performed in a subset of patients (n=53) to assess luminal status. PAM50 results analysis showed that patients with Luminal B tumours had a significantly higher anti-proliferative response with the combination of anastrozole plus pictilisib compared to anastrozole alone [geometric mean Ki67 suppression, 86.5% versus 63.6%; ratio (combination/anastrozole) 0.37 (0.18-0.76; p=0.008)], whereas adding pictilisib to anastrozole had no apparent benefit for Luminal A tumours (ratio, 1.01; p=0.98).

Baseline Ki67 expression, PR, tumour grade and treatment response:

As it had been hypothesised that luminal B biology could be a determinant of suboptimal response to endocrine therapy alone and potentially therefore define a subgroup that might derive an increased benefit from combination therapy with pictilisib and anastrozole, the impact of a number of baseline characteristics that have been linked with luminal B phenotype were explored. These include baseline Ki67 expression, PR expression and tumour grade.

In an analysis involving all evaluable patients (n=136), luminal status was defined by baseline Ki67 expression in accordance to the St Gallen criteria (Coates et al., 2015) using a Ki67 expression of 14% as the cut-off between luminal A and luminal B. In contrast to the PAM50 analysis (Figure 15a), patients with Luminal A status (n=50) defined as baseline Ki67 of <14% had a significant benefit of the combination of anastrozole plus pictilisib compared to anastrozole alone [geometric mean Ki67 suppression, 74.1% versus 43.4%; ratio (combination/anastrozole) 0.46 (0.25 – 0.85); p=0.02)] (Figure 15b). In patients with Luminal B tumours (n=86), defined as Ki67 >14%, geometric mean Ki67 suppression was 78.7% in the anastrozole alone group and 86.3% for patients treated with anastrozole plus pictilisib [ratio, 0.64 (0.43 – 0.97); p=0.04].

Using a Ki67 cut-off of 20%, mean geometric Ki67 suppression for Luminal A tumours was 61.6% in the anastrozole alone group and 77.6% for patients treated with anastrozole plus pictilisib [ratio, 0.58 (0.25 – 0.97); p=0.04]. For Luminal B tumours, geometric mean Ki67 suppression was 77.6% for patients treated with anastrozole

alone and 86.7% for patients treated with anastrozole plus pictilisib [ratio, 0.59 (0.36 - 0.96); p=0.04].

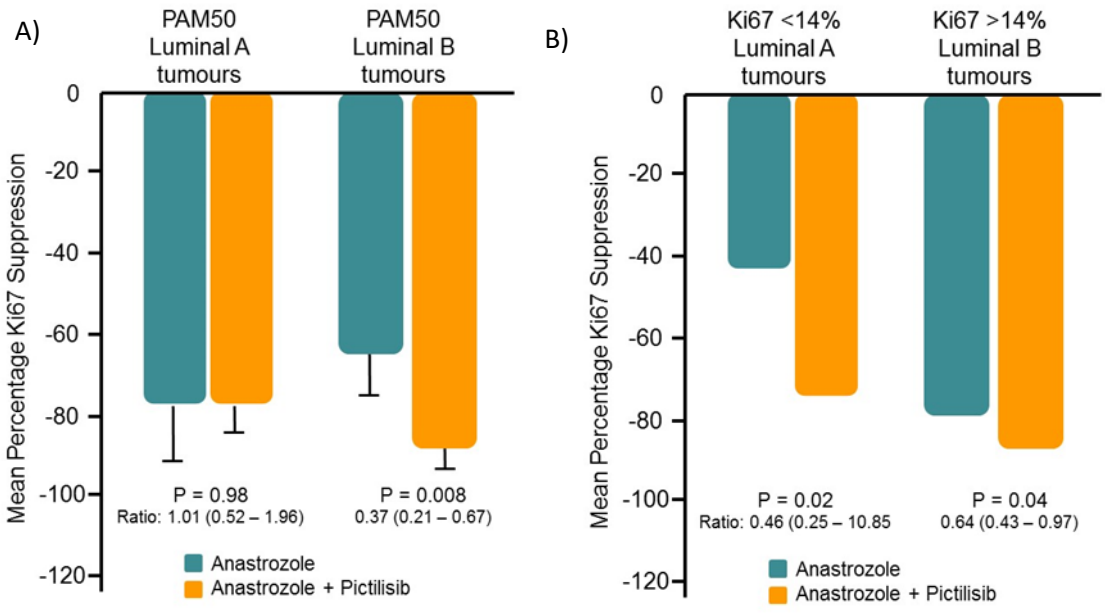


Figure 15: Anti-proliferative response to study treatment; A) anti-proliferative response by Luminal subtype defined by PAM50; B) anti-proliferative response by Luminal subtype defined by baseline Ki67 expression (cut-off 14%)

PR receptor status was available in all 136 patients; the majority of tumours were classified as PR positive (84.6%), defined by an Allred score of 3 or higher (Figure 16A). Only 21 tumours were PR negative (15.4%). The addition of pictilisib increased the anti-proliferative response in both subsets with a slightly more pronounced benefit in patients with PR negative tumours. In PR-positive tumours, the geometric mean Ki67 suppression was 72.1% with anastrozole compared to 81.7% with the combination [0.65 (0.43–0.98); p=0.04], whereas in PR-negative tumours the mean

Ki67 suppression was 66.7% with anastrozole compared to 88.4% with the combination [0.35 (0.14–0.87); p=0.03] (Figure 16a).

Tumour grade was available for 135 patients; the majority of tumours were classified as Grade 1 or Grade 2 (n=115; 85.2%) with the remaining 21 tumours classified as Grade 3 (15.6%). Tumour grade was a strong predictor of response to anastrozole alone with a mean geometric Ki67 suppression of 73.2% (61.0%-81.6%) in patients with Grade 1 or Grade 2 tumours compared to 50% (19.4%-69.0%) in patients with G3 tumours (Figure 16B). In contrast, patients responded to anastrozole plus pictilisib irrespective of the tumour grade with a mean geometric Ki67 suppression of 80.4% (74.8%-84.8%) for patients with Grade 1 or Grade 2 tumours and 90.3% (78.8%-95.5%) for patients with Grade 3 tumours.

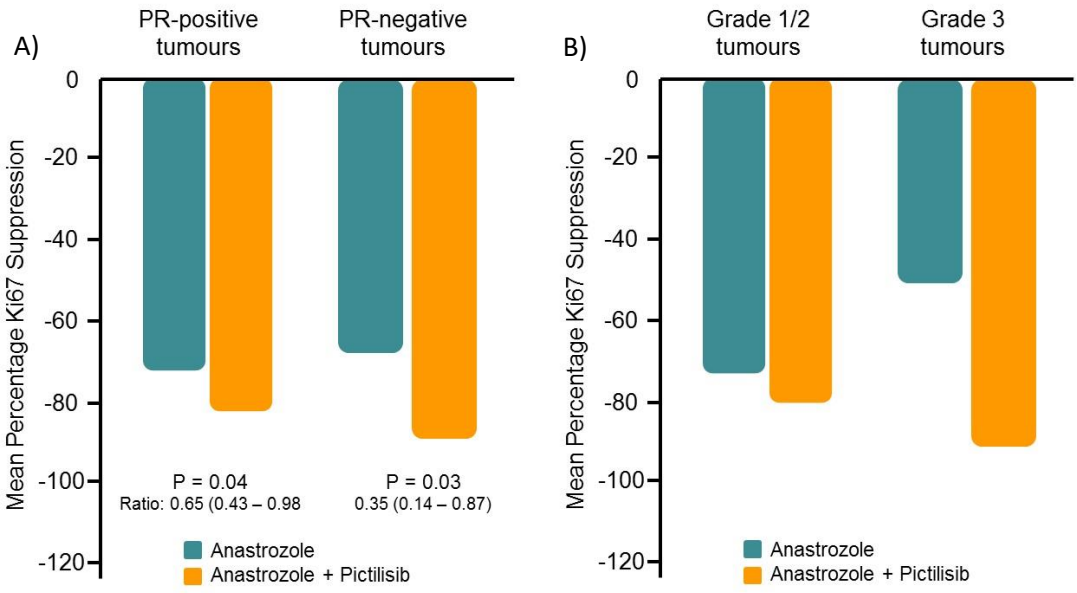


Figure 16: Anti-proliferative response to study treatment PR status (a) and tumour grade (b)

Multivariate linear regression analysis confirmed a significant interaction between treatment effect and molecular subtype by PAM50 (p=0.03), supporting the observation that the combination treatment is more effective than anastrozole alone for patients with Luminal B tumours irrespective of PR status or the baseline Ki67 expression. However, patients with PR-negative Luminal B cancers showed the greatest anti-proliferative effect from combination treatment (ratio=0.12). Furthermore, combined treatment also appeared to be more effective in PR-negative Luminal A cancers.

4 PI3K kinase pathway activation and treatment benefit

To further assess the potential interaction of activation of the PI3K pathway and study treatment, we performed analysis of changes in gene/protein expression and phosphorylation of selected signalling markers.

Two gene expression signatures (GS) were calculated at baseline and at the end of treatment using the Nanostring gene expression data. The GS data were correlated with baseline characteristics and response to anastrozole and the combination therapy, respectively. The PIK3CA mutation associated GS (Loi et al., 2010) has previously been shown to negatively correlate with proliferation, AKT/mTOR activation and PTEN loss and strongly positively correlated with ESR1 and better outcome in ER-positive breast cancer. O'Brien et al identified a PIK3 inhibitor sensitivity GS, based on a number of genes that are differentially expressed between sensitive and resistant breast cancer cell lines; the PIK3 inhibitor sensitivity GS (O'Brien et al., 2010) has been shown to correlate with activation of the PI3K pathway and can be used to characterise patients who are sensitive to PI3K inhibition.

The baseline PIK3 inhibitor sensitivity GS (O'Brien) score was associated with higher proliferation and Luminal B phenotype (Figure 17a). Post-treatment PIK3 inhibitor sensitivity GS (O'Brien) scores were significantly down-regulated in both arms, consistent with an attenuation of the flux through the PI3K pathway (Figure 17b).

In contrast, we observed no relevant modulation of the PIK3CA mutation-associated GS (Loi) with study treatment (Figure 17c). The PIK3CA mutation-associated GS (Loi) was not predictive of a treatment-induced change in Ki67 in either treatment arm.

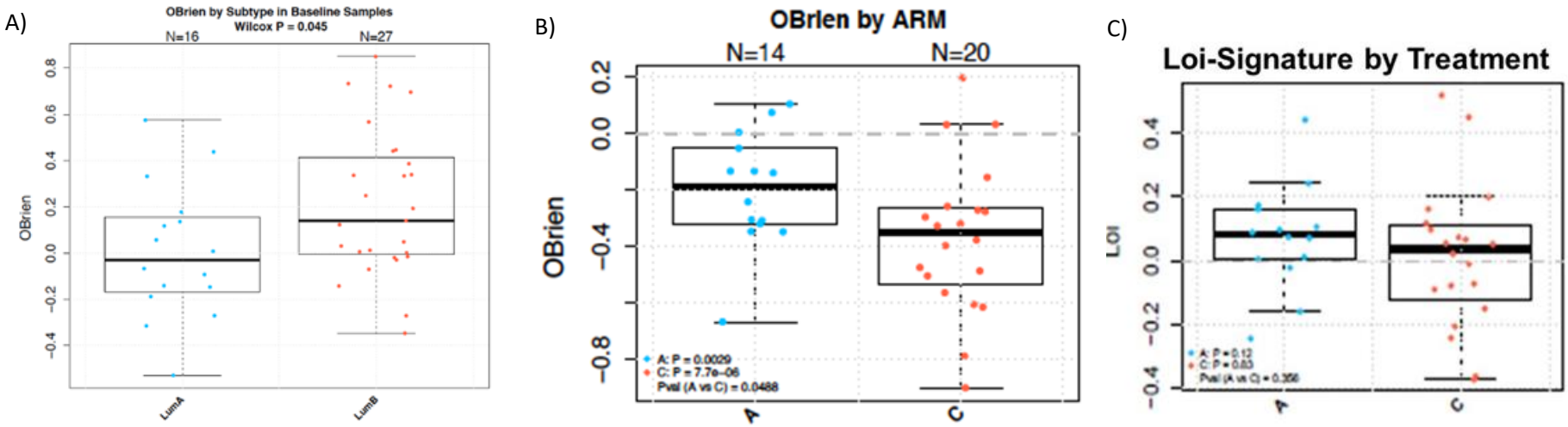


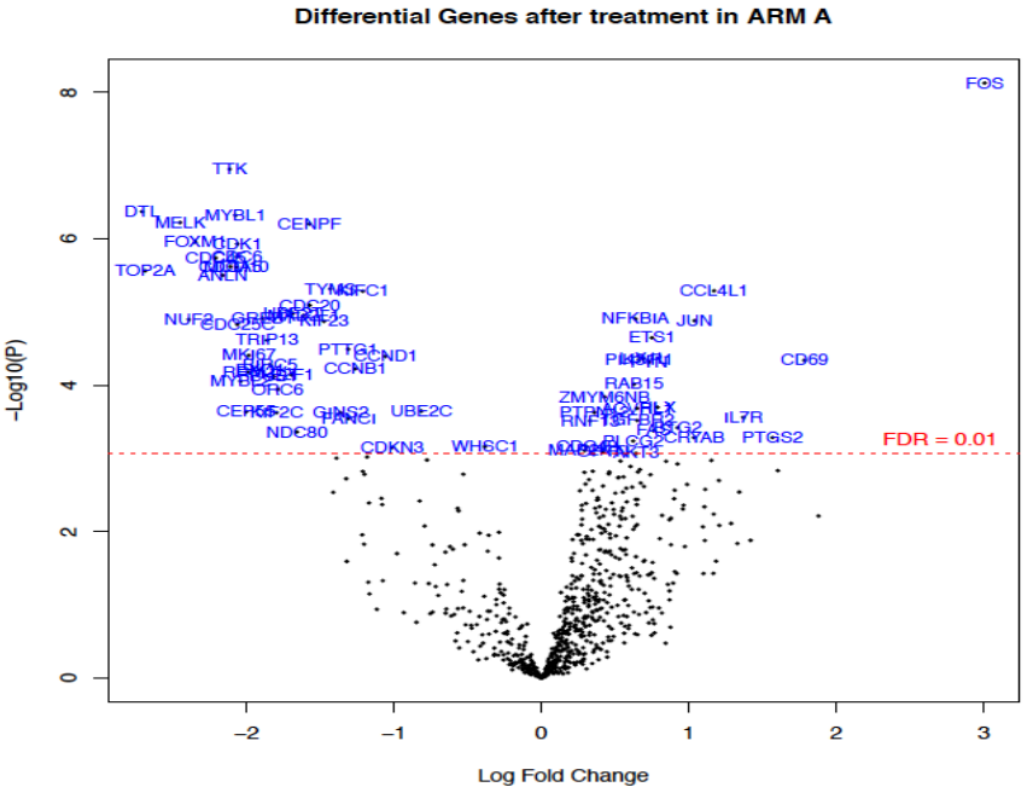
Figure 17: PI3K mutation and PI3K inhibitor sensitivity gene signatures: a) association of baseline PIK3 inhibitor sensitivity GS (O'Brien) score and Luminal B phenotype; b) down-regulation of post-treatment PIK3 inhibitor sensitivity GS (O'Brien) scores in both treatment arms; c) post-treatment PIK3CA mutation-associated GS (Loi) in both treatment groups; A=anastrazole only, C=combination with pictilisib.

5 Treatment-induced changes in gene/protein expression and phosphorylation

Treatment-induced changes in protein expression and phosphorylation and gene expression were evaluated in subsets of patients, using RPPA (n=32) and Nanostring analysis (n=64). Figure 18a and b provide an overview of differentially expressed genes between pre- and post-treatment samples in the anastrozole and anastrozole and pictilisib groups, respectively. The top ten genes differentially expressed in each arm of the treatment groups are shown in Table 8. The top differentially expressed canonical pathways in the anastrozole group included cyclines and cell cycle regulation, oestrogen-dependent signalling and gene expression, ATM signalling, mitotic kinases, and aryl hydrocarbon receptor signalling. There was substantial upregulation of a number genes associated with the immune system, whereas many of the most downregulated genes were involved in cell cycle control.

In the anastrozole plus pictilisib group, top upregulated canonical pathways included pancreatic adenocarcinoma signalling, aryl hydrocarbon receptor signalling, IL-8 signalling, bladder cancer signalling and GADD45 signalling. There was also substantial upregulation of genes associated with the immune system and downregulation of cell cycle genes.

A)



B

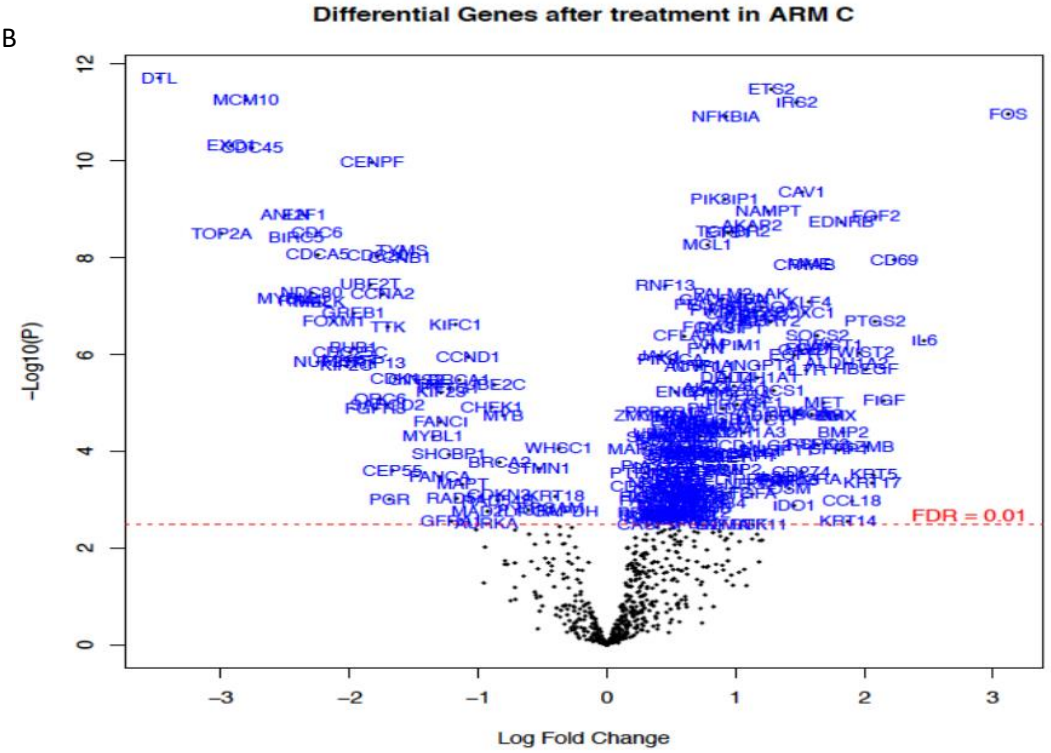


Figure 18 Differentially-expressed genes between pre- and post-treatment samples in the anastrozole arm (a) and anastrozole and pictilisib arm (b)

A)

	Log2 fold change	std error (log2)	P-value	probe.ID
RAB7B-mRNA	3.6	0.622	1.98E-06	NM_177403.3:1485
Fos-mRNA	3.45	1.01	0.0018	NM_005252.2:1475
TWIST1-mRNA	3.2	0.606	8.81E-06	NM_000474.3:35
SRC2-mRNA	2.98	0.628	4.17E-05	NM_005248.1:375
eif4b-mRNA	2.63	0.676	0.000471	NM_001417.4:295
CCL4L1-mRNA	2.55	0.452	3.07E-06	NM_001001435.2:11
CD68-mRNA	2.51	0.629	0.000366	NM_001251.2:1140
RPS6-mRNA	2.45	0.593	0.000241	NM_001010.2:171
FOXA1-mRNA	2.45	0.675	0.000957	NM_004496.2:2465
CCL18-mRNA	2.41	1.05	0.0286	NM_002988.2:585
MAD2L1-mRNA	-1.09	0.433	0.0165	NM_002358.3:182
ORC6L-mRNA	-1.1	0.457	0.0225	NM_014321.2:582
CDC20-mRNA	-1.1	0.508	0.0382	NM_001255.2:430
UBE2T-mRNA	-1.14	0.381	0.00518	NM_014176.3:595
E2F1-mRNA	-1.26	0.509	0.0187	NM_005225.1:935
CCND1-mRNA	-1.28	0.531	0.0217	NM_053056.2:690
TRIP13-mRNA	-1.47	0.494	0.00556	NM_004237.2:450
DTL-mRNA	-2.01	0.454	0.000106	NM_016448.2:380
FGFR3-mRNA	-2.04	0.624	0.00263	NM_022965.2:3170
GREB1-mRNA	-2.29	0.564	0.000289	NM_014668.3:1360

B)

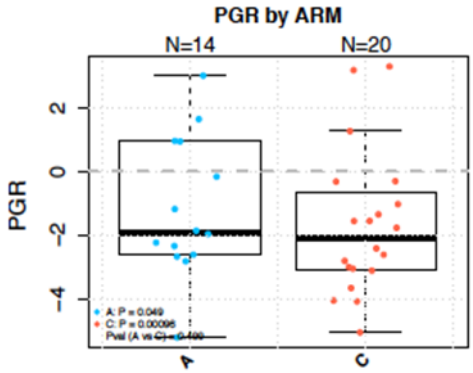
	Log2 fold change	std error (log2)	P-value	probe.ID
Fos-mRNA	3.86	0.655	8.32E-08	NM_005252.2:1475
Jun-mRNA	3.45	0.44	1.62E-11	NM_002228.3:140
MAML2-mRNA	2.51	0.448	2.99E-07	NM_032427.1:4125
CD11c-mRNA	2.31	0.445	1.61E-06	NM_000887.3:700
Decorin-mRNA	2.12	0.439	6.88E-06	NM_001920.3:420
ALDH1A1-mRNA	2.08	0.441	1.02E-05	NM_000689.4:276
IRS2-mRNA	2.02	0.389	1.57E-06	NM_003749.2:775
MYC-mRNA	1.99	0.45	3.03E-05	NM_002467.3:1610
ALDH1A3-mRNA	1.97	0.426	1.48E-05	NM_000693.2:2280
ETBR-mRNA	1.94	0.402	6.84E-06	NM_003991.2:560
S100A14-mRNA	-2.34	0.599	2.00E-04	NM_020672.1:460
FGFR3-mRNA	-2.38	0.6	0.000157	NM_022965.2:3170
Pfs2-mRNA	-2.76	0.447	2.62E-08	NM_016095.2:990
GFRA1-mRNA	-2.82	0.651	4.22E-05	NM_005264.4:1895
PGR-mRNA	-2.89	0.518	3.03E-07	NM_000926.2:3165
UBE2C-mRNA	-2.92	0.401	2.10E-10	NM_181803.1:269
GREB1-mRNA	-3.16	0.391	5.54E-12	NM_014668.3:1360
CENPF-mRNA	-3.48	0.419	2.11E-12	NM_016343.3:5822
BIRC5-mRNA	-3.51	0.43	3.96E-12	NM_001168.2:1215
RRM2-mRNA	-3.96	0.441	9.69E-14	NM_001034.1:1615

Table 8 The top 10 gene transcripts overexpressed and repressed in A) anaztrozole and B) combination treated patients. Only genes that with p-value <0.05 are included.

ER target genes: Previous data suggested that single agent PI3K inhibition up-regulates expression of ER target genes in vivo and in vitro (Bosch et al., 2015). In a preclinical study, treatment with the PI3K inhibitor BYL719 (p110a) was associated with substantially increased expression of ER-target genes. Furthermore, treatment with BYL719 upregulated ESR1 expression in tumour samples of treated patients.

We therefore investigated the effect of treatment with pictilisib and anastrozole on ER target genes, using Nanostring gene expression analysis. As illustrated in Figure 19 there was a significant treatment-associated reduction in the expression of ER target genes such as GREB1 or PR. No differences were observed between the 2 study arms, suggesting that induction of ER target genes by PI3K inhibition requires oestrogen.

A)



B)

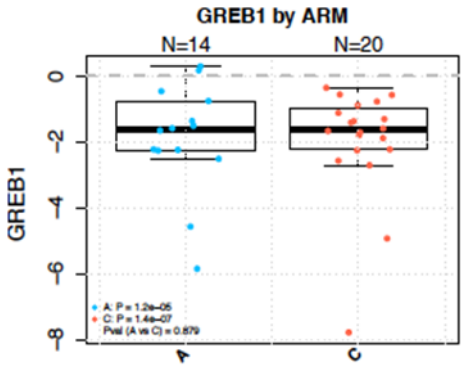


Figure 19: Treatment-induced changes in expression of ER target genes PR and GREB1; A, anastrozole alone; C, combination.

PI3K pathway and cell cycle. RPPA analysis focused on key genes involved in the activation of the PI3K pathway and cell cycle. Baseline protein expression and phosphorylation was comparable between both groups. There was substantial downregulation of cell cycle genes in both arms, associated with endocrine therapy. Although total AKT levels seemed to increase more in anastrozole-treated patients, end-of-treatment Phospho-AKT levels were similar in both treatment groups. Similarly, ESRa levels appeared higher in the anastrozole group but phospho-ESRa

was comparable. RPPA levels suggest a possible increase in Phospho-Raptor levels with anastrozole but pS6 levels or p4E-BP1 levels were similar between both arms (Figure 20; Figure 21). Overall, the end-of-treatment profiles as well as the treatment-associated changes (Figure 21) were largely comparable between both groups, suggesting a dominant anti-oestrogen effect. The effects on Cyclin D1 were more pronounced with the combination in keeping with the more substantial anti-proliferative effect as per Ki67 analysis.

Gene expression analysis in the anastrozole group demonstrated upregulation of genes involved in cell cycle arrest such as p21. In the combination group, gene expression analysis also demonstrated up-regulation of the PI3K regulated genes IRS2 and PIK3IP1, confirming treatment-associated pathway inhibition (Figure 20).

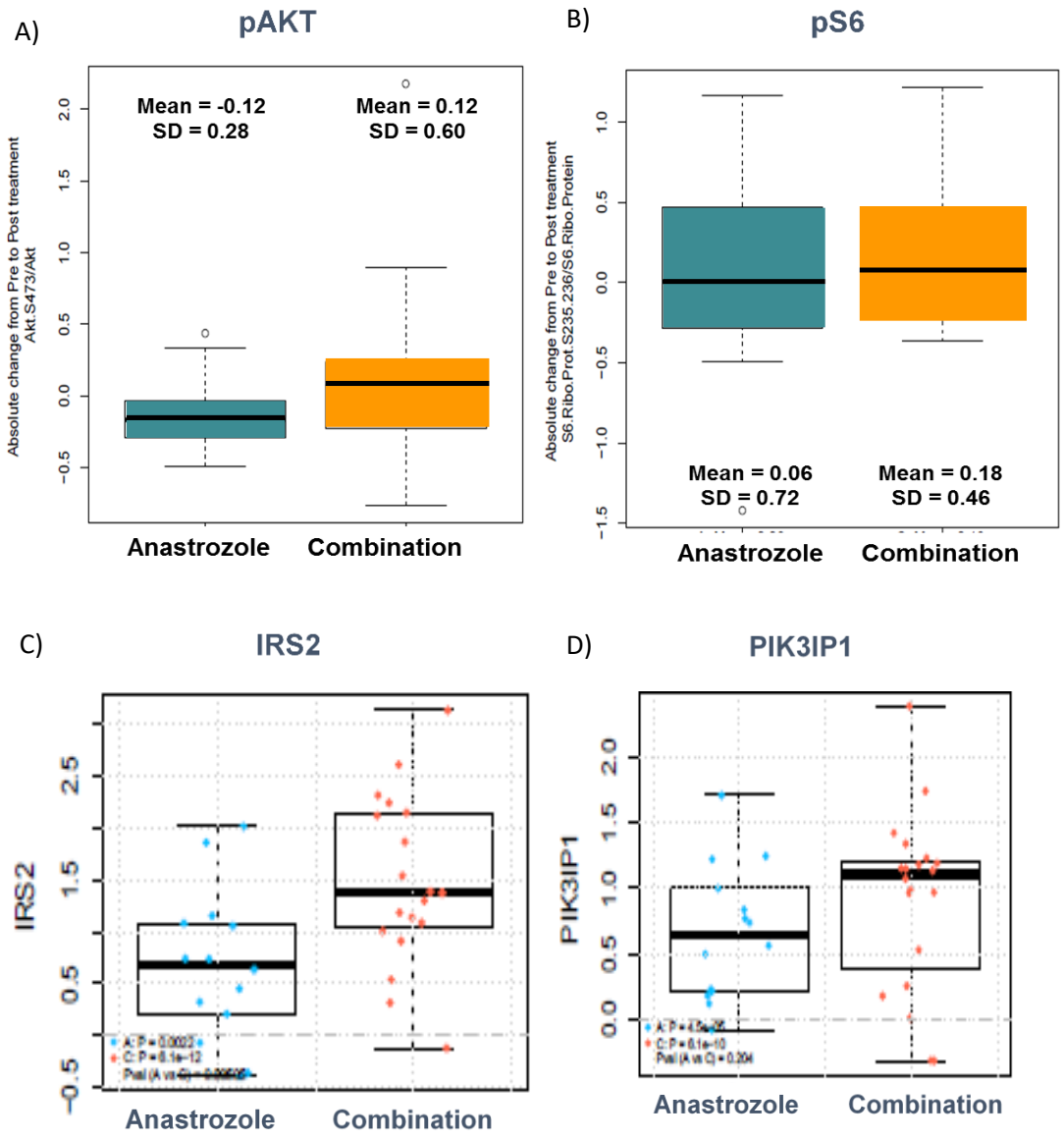


Figure 20: RPPA analysis of Phospho-AKT levels (a) and pS6 levels (b) after treatment with anastrozole or combination therapy; treatment-associated upregulation of PIK3CA-regulated genes PIK3IP1 and IRS2

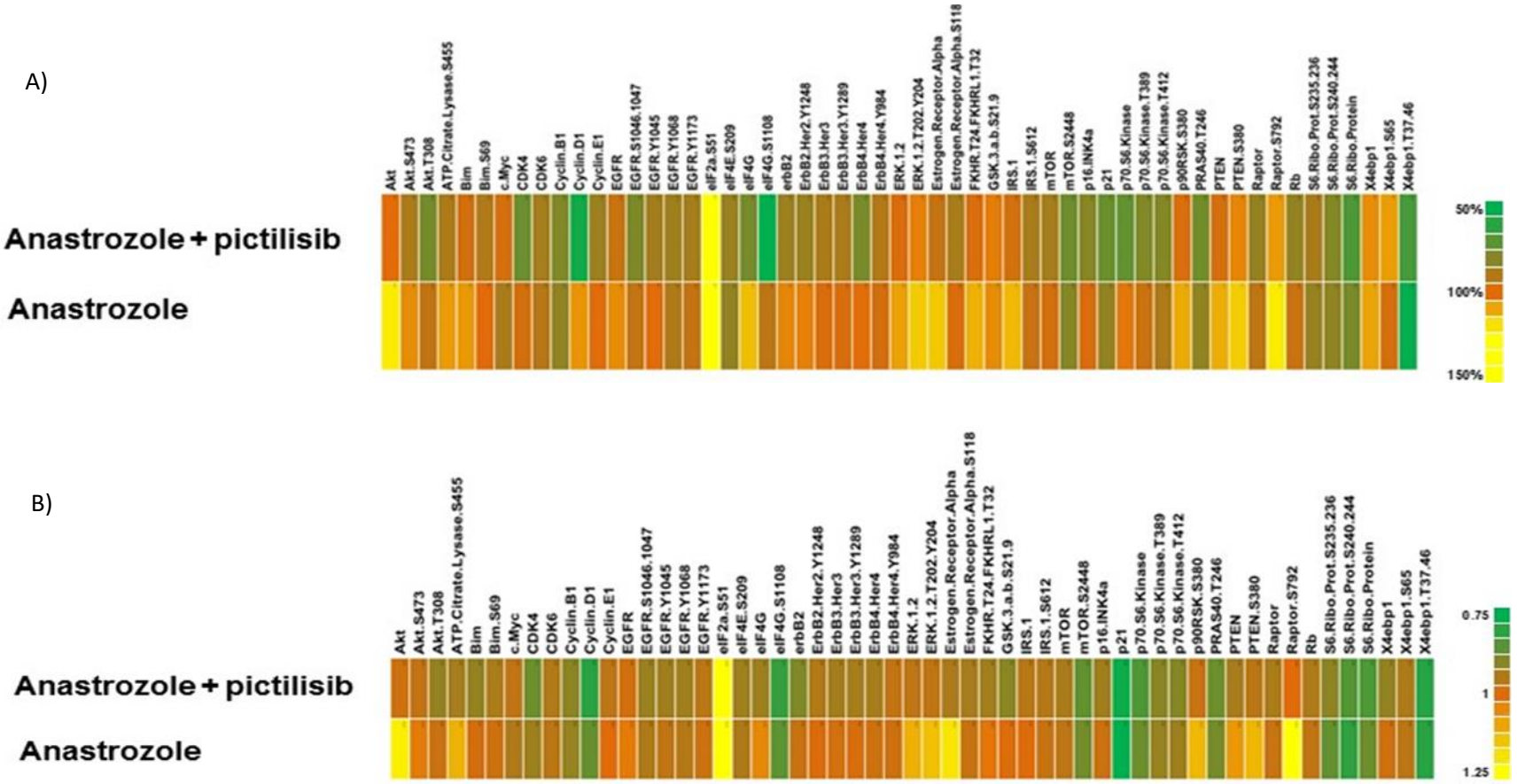


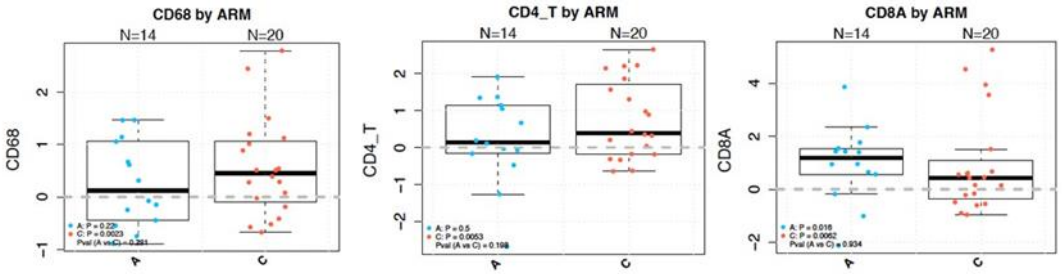
Figure 21: RPPA analysis focusing on key genes involved in the activation of the PI3K pathway and cell cycle. A) Mean end-of-treatment RPPA expression in the anastrozole and combination therapy groups; B) Mean treatment-associated changes in RPPA expression with anastrozole and anastrozole plus pictilisib

6 Effects of PI3K inhibition on the tumour microenvironment and immune system

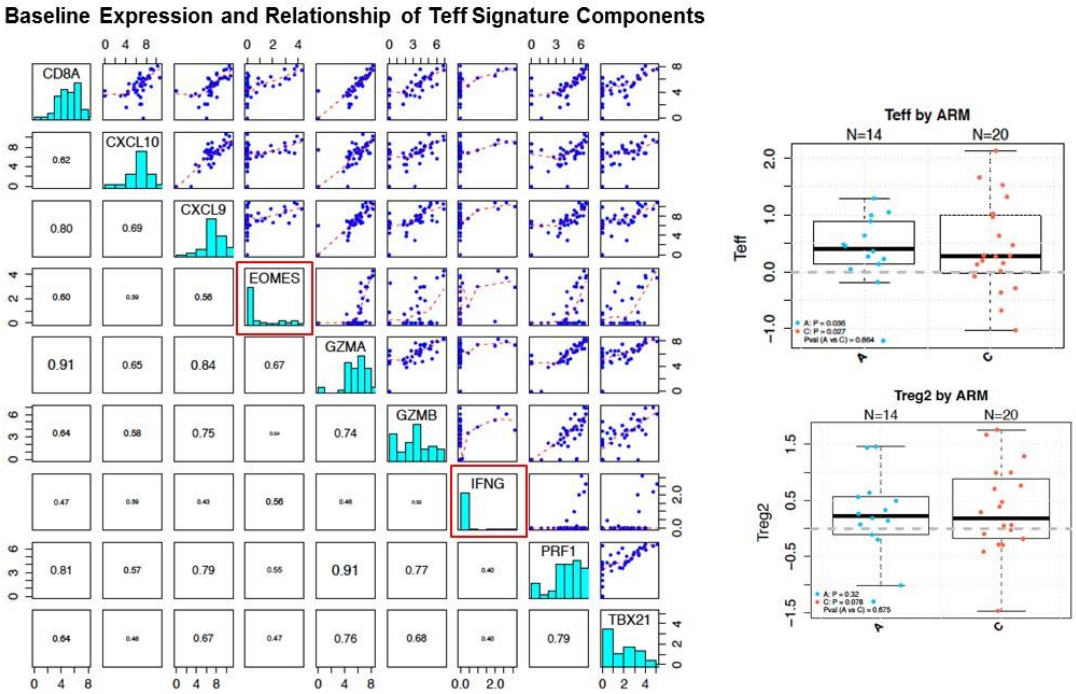
There is substantial evidence that E2 and/or ER signalling regulates the development and function of dendritic cells (Kovats, 2012), B and T lymphocytes, NK cells, monocytes and macrophages (Cunningham and Gilkeson, 2011; Straub, 2007). In addition, there is discussion around the role of PI3K signalling and the tumour microenvironment. There are examples of increasing nonclinical and clinical data suggesting a correlation between PTEN loss and impaired anti-tumour immune response, including reduced CD8 T-cell infiltration and reduced efficacy of anti-PD1 therapy. Furthermore, nonclinical studies reveal synergistic anti-tumour responses when combining PI3K/AKT pathway inhibition and PD-L1/PD-1 axis blockade (Peng et al., 2016). In addition, PI3K or AKT inhibitors may restore and enhance physiological functionalities of T cells in the tumour microenvironment and enhance expansion of tumour-specific lymphocytes with memory cell phenotype (Crompton et al., 2015). Treatment with AKT inhibitors has also been shown to promote the development of memory T cells over effector T cells (Gubser et al., 2013; Xue et al., 2015). Based on these findings, we therefore performed gene expression analysis to assess the impact of anastrozole and the combination therapy on the tumour microenvironment and immune system. The major gene transcript representative for specific immune cells were examined if there were any changes. Analysis of pre and post treatment samples showed that a 2-week treatment of anastrozole and anastrozole plus pictilisib have a modest impact on the tumour immune microenvironment; the observed effects differed between the two treatment groups (Figure 22a). Whilst in

patients treated with anastrozole a modest increase in CD8A transcript was observed, combination therapy was associated with a modest increase in CD68, CD4 and CD8A transcripts. In the tumour samples from patients that received combination therapy, there was an increase in the expression of markers indicative for macrophages, CD4 and CD8+ cell recruitment, as well as increase of immunosuppressive molecules such as PD-L1, PD-L2 and IDO (Figure 22b). No significant increase in FOXP3+ effector cells was observed in either arm. Study treatment had a minimal impact on T_{eff} and T_{reg} signatures and on T-cell immunosuppressive signature but a modest impact on APC immunosuppressive signature (Figure 22c, d).

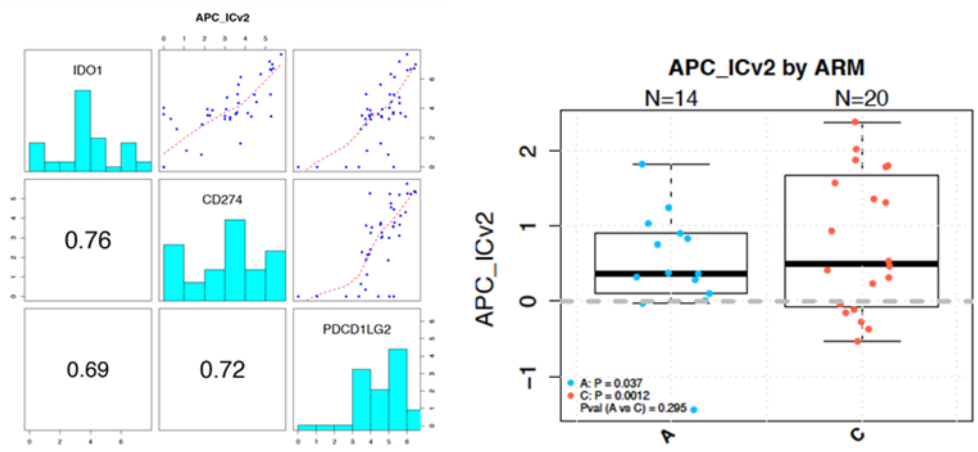
A)


CD68 by ARM
N=14
N=20
CD68
A
C
CD4_T by ARM
N=14
N=20
CD4_T
A
C
CD8A by ARM
N=14
N=20
CD8A
A
C

B)


Baseline Expression and Relationship of Teff Signature Components
CD8A
CXCL10
CXCL9
EOMES
GZMA
GZMB
IFNG
PRF1
TBX21
Teff by ARM
N=14
N=20
Teff
A
C
Treg2 by ARM
N=14
N=20
Treg2
A
C

C)



D)

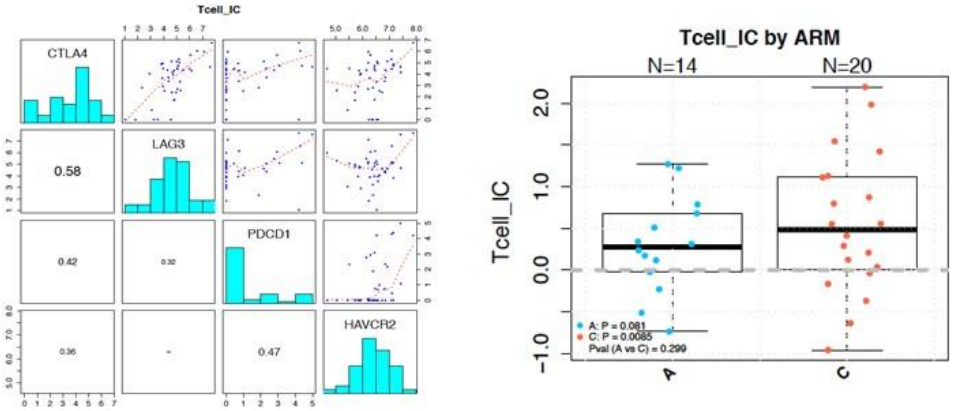


Figure 22 a) Impact on markers of immune cell populations (CD68, CD4, CD8A) in the post-treatment samples; b) Treatment effect on T_{eff} and T_{reg} signatures; c) APC immunosuppressive signature; d) T-cell immune-suppressive signature

Chapter 5 Discussion

The OPPORTUNE trial was designed to evaluate whether the addition of the PI3K inhibitor pictilisib to endocrine therapy with anastrozole can increase the effects on tumour cell proliferation or apoptosis, to identify potential predictors of sensitivity to PI3K-inhibition, and to study the effects of combined endocrine and PI3K-inhibitor therapy on breast cancer biology.

The primary endpoint of the study was to assess treatment-associated changes in the expression of the proliferation marker Ki67 after two weeks of preoperative endocrine therapy. Short-term, preoperative studies of 2-week therapy are a validated strategy to evaluate the impact of targeted therapies alongside endocrine agents, using the nuclear proliferation marker Ki67 as an intermediate surrogate endpoint of treatment benefit (Dowsett et al., 2007, 2005; Ellis et al., 2008; Hadad et al., 2011; Macaskill et al., 2011; Polychronis et al., 2005). The study required each site to fix each sample for more than 6 hours and embed the tumour samples locally before being assessed at a central site. Due to the nature of the multi-center trial, it was not possible to remove any variation in the pre-analytical steps, but the variation is removed by cutting, staining and assessing at a central site (Yerushalmi et al., 2010). In this study, Ki67 expression was assessed centrally by IHC according to previously established and validated criteria and expression was recorded independently by two investigators, who were blinded as to treatment allocation and each other's assessment (Prof Sarah Pinder, Dr Louise Lim). We were able to demonstrate high concordance between both Ki67 assessments. The mean (median) difference between the 2 analyses was 3.90% (3.06%) for the baseline assessment

and 2.63% (1.72%) for the end of treatment analysis, respectively. The Cohen's kappa value represents the degree of accuracy and reliability in statistical classification. When using the defined 14% cut-off, the kappa value of 0.77 found substantial agreement between the independent scorers. The cut-off value for Ki67 is currently debatable despite the number of published studies reporting significant results, ranging from 1-30% (Dowsett et al., 2011). We have decided on 14% for Ki-67 is as recommended by the St Gallen panel to differentiate between low proliferation (<14%) and high proliferation tumours (Goldhirsch et al., 2011). As defined in the statistical analysis plan, all endpoint analyses were performed using the geometric mean of the two Ki67 assessments. Our results demonstrate that adding pictilisib to anastrozole significantly increased the anti-proliferative response compared with two-week preoperative anastrozole treatment in previously untreated patients with ER-positive invasive breast cancer as measured by suppression of Ki67 expression, confirming the primary trial hypothesis. As previously established, we applied different analytical methods to assess the treatment-associated changes in Ki67 expression. Mean Ki67 suppression was selected as the primary analysis method in keeping with previous pivotal trials in this area. Ki67 suppression was calculated after log transformation of Ki67 values, assuming a log normal distribution. Results were subsequently back transformed to their original scale. We were able to demonstrate a statistically significant reduction in mean percentage suppression of Ki67 from 70.7% (61.0%-78.0%) for anastrozole treated patients to 82.5% (95% CI, 78.3%-85.8%) for anastrozole plus pictilisib treated patients, which is an approximately 40% increased suppression (ratio, 0.60 0.58-0.85; p=0·01).

An alternative method for the Ki67 analysis focused on end of treatment Ki67 expression. This endpoint is particularly relevant as reduced Ki67 expression after two weeks of preoperative endocrine therapy has been associated with an improved recurrence-free survival (RFS) in ER positive postmenopausal breast cancer patients (Dowsett et al., 2007). In the preoperative IMPACT trial, which evaluated 4 months of preoperative therapy with anastrozole, tamoxifen, or anastrozole plus tamoxifen in ER-positive, postmenopausal BC patients but also had an early assessment of Ki67 changes after 2 weeks, the hazard ratio for RFS was 1.95 (95% CI 1.23-3.07) per 2.7-fold increase of Ki67 expression after two weeks of preoperative endocrine therapy (Dowsett et al., 2007). 5-year RFS rates were 85%, 75%, and 60%, respectively, for the lowest, middle, and highest tertiles of end of treatment Ki67 expression (Dowsett et al., 2007). In contrast, baseline Ki67 expression was only predictive of outcome on univariate analysis but not on multivariate analysis (HR 1.09; 95% CI 0.60-1.99), suggesting that measurements of tumour cell Ki67 expression after two weeks of endocrine treatment may integrate the intrinsic prognostic value of baseline Ki67 expression measurements and the predictive value of the treatment–determined changes in Ki67 expression (Dowsett et al., 2007). Interestingly, the results of the IMPACT trial, which demonstrated an increased anti-proliferative response to anastrozole compared to tamoxifen or anastrozole plus tamoxifen, were highly concordant with the results of the large adjuvant ATAC trial (Arimidex, Tamoxifen, Alone or in Combination (ATAC) Trialists' Group et al., 2008).

Similar results were obtained in the POETIC phase III trial, which was set up to determine whether 2 weeks perioperative aromatase inhibitor (AI) therapy before and after surgery improves outcome compared with standard adjuvant therapy alone

in postmenopausal women with ER/PR positive invasive breast cancer (Robertson et al., 2018). The trial used Ki67 expression as a primary measure of the anti-proliferative effects of the study treatment and correlated it with long term outcome. A total of 4,486 patients were recruited from 130 UK sites over a 5.5-year period. Patients with baseline Ki67 expression of <10%, generally had an excellent outlook with a 5-year absolute recurrence risk of 4.5% (95% CI, 3.1%-6.6%). More importantly, in patients with a baseline KI67 expression of ≥10%, the end of treatment Ki67 expression was a strong predictor of long term outcome [HR 2.22 (95%CI: 1.68, 2.94); p<0.001]. The 5-year absolute recurrence risk was 8.9% (7.2%-11.0%) for patients with end of treatment Ki67 levels of <10% compared to 19.6% (15.9%-24.1%) with end of treatment KI67 expression levels of ≥10%.

As previously established, we used 2 different ways of analysing end of treatment response either through the geometric mean Ki67 expression at the end of study treatment (Mean $Ki67_{post}$) or the EOT response rate RKi67-Day15 defined as the natural logarithm of percentage Ki67 positive cells of less than 1 or 1-2 at the end of study treatment. Both the geometric mean end of treatment Ki67 suppression and the EOT response rate RKi67-Day15 were increased with the combination of anastrozole plus pictilisib compared to anastrozole alone, further supporting the main hypothesis that the addition of the PI3K inhibitor improves the anti-proliferative effects of anastrozole.

The second main hypothesis was whether combining endocrine therapy and PI3K inhibitors can lead to an increase in tumour cell apoptosis. This was supported by preclinical data demonstrating that oestrogen can suppress apoptosis induced by

PI3K inhibition in ER-positive breast cancer, suggesting independent PI3K- and E2-dependent cell survival mechanisms. As a result, combination of endocrine therapy and PI3K inhibitors can lead to synthetic lethality with substantially increased apoptosis compared to single-agent therapy. We therefore evaluated changes in tumour cell apoptosis, as measured by Caspase 3 expression in pre and post-treatment tumour samples, between both treatment groups. As for Ki67, caspase-3 expression was assessed centrally by IHC according to previously established and validated methods and expression was recorded independently by two investigators who were blinded as to treatment allocation and each other's assessment (Prof Sarah Pinder, Dr Louise Lim). We were again able to demonstrate high concordance between both caspase-3 assessments.

In keeping with other studies in this field, the rate of apoptosis was low in this trial with the majority of tumour samples containing less than 1% apoptotic cells and a geometric mean expression of 0.15%. There was no clear evidence of a treatment-associated increase in Caspase-3 expression but the results have to be interpreted with caution, as the low rate of apoptosis together with the strong positive correlation between Ki67 and apoptosis scores, found in this and other trials (Dowsett et al., 2007), could mask an effect of PI3K inhibition on apoptosis as observed in preclinical studies (Crowder et al., 2009). Other groups have therefore introduced growth index, defined as percent Ki67-expresion divided by percent Caspase-3 expression. Using this method, we were able to describe a greater suppression in the growth index in the combination arm (75.2%) compared to anastrozole alone 55.9%, although this was not statistically significant.

The third main objective of this thesis was to investigate the interaction between the treatment benefit of pictilisib and/or anastrozole and mutations of PIK3CA, the gene encoding the catalytic PI3K subunit p110α, the target of pictilisib. Three major hotspots of mutations of the PIK3CA gene have been described; these are concentrated in the helical (E542K and E545K) and kinase (H1047R) domains, accounting to approximately 90% of all PIK3CA mutations. In keeping with other series in ER-positive primary breast cancer [4,8], 36% of the tumours in our study carried at least mutation in the PIK3CA gene, 98% of these in one of the three major hotspots in the helical (E542K and E545K) and kinase (H1047R) domains.

Taking all types of PIK3CA mutations together, there was no association between overall PIK3CA mutation status and anti-proliferative response for anastrozole alone, in keeping with other studies suggesting that the presence of PIK3CA mutations has limited impact on the effect of preoperative anastrozole therapy in patients with primary, ER-positive breast cancer (Krop et al., 2016; López-Knowles et al., 2014; Ma et al., 2017). There was also no correlation between overall PIK3CA mutation status and added activity of pictilisib with a ratio of geometric mean Ki67 proportional change of 0.63 (0.39–1.0) for patients with PIK3CA-wildtype tumours and 0.72 (0.46–1.15) for patients with PIK3CA-mutated tumours. This is consistent with results from trials of pictilisib or the mTOR inhibitor everolimus in pre-treated, metastatic breast cancer, where patients derived benefit from everolimus or pictilisib regardless of their tumour PIK3CA genotype (Hortobagyi et al., 2016a; Krop et al., 2016; Treilleux et al., 2015). However, recent trials with more specific PI3K inhibitors (α-specific or β-sparing), have demonstrated an increased benefit in patients with PIK3CA mutated tumours (SOLAR1, SANDPIPER) (André et al., 2019; Baselga et al., 2018). This might

be down to more profound target inhibition in the tumour which might be achievable due to the improved efficacy/tolerability ratio associated with the lower degree of β-inhibition which has been associated with driving toxicity.

Interestingly, we found a significant interaction between PIK3CA mutation subtypes and Ki67 suppression. Whilst patients with helical domain mutations [ratio, 0.48 (0.27-0.84; p=0.02] or PIK3CA wildtype status [ration, 0.63 (0.39–1.0; p=0.05] demonstrated a substantial relative benefit from the addition of pictilisib, there was not clear additional effect of pictilisib in tumours with KD mutations [ratio, 1.17 (0.57–2.41; p=0.64]. This was largely due to patients with HD mutations showing a particularly poor response to anastrozole alone [mean Ki67 suppression 53.9% (9.5%-76.5%)], that was reversed by the addition of pictilisib [mean Ki67 suppression, 78.1% (71.0%-83.4%)], whereas patients with KD mutations responded well to anastrozole alone [mean Ki67 suppression 77.7% (57.0%-88.4%)] and showed no benefit from the addition of pictilisib [mean Ki67 suppression 73.9% (59.8%-83.0%)]. A similar observation was reported from a neoadjuvant trial of the mTOR inhibitor everolimus and letrozole in ER-positive breast cancer, in which exon 9 mutations seemed to be associated with an increased benefit of mTOR inhibition relative to exon 20 mutations (Baselga et al., 2009) and this may merit testing in future studies of early breast cancer.

PTEN expression was not associated with benefit of the combination therapy and also did not add significantly to PIK3CA mutations as determinant of PI3K inhibitor benefit. As expected being associated with PIK3CA mutations rather than PI3K pathway activation, the Loi GS did not change significantly with study treatment and

was not predictive of a treatment-associated change in Ki67 expression in either treatment arm. On the other hand, the O'Brien GS, which was developed based on a number of genes that are differentially expressed between sensitive and resistant breast cancer cell lines, was significantly down-regulated with both study treatments, consistent with an attenuation of the flux through the PI3K pathway. It was also inversely associated with Ki67 suppression in the anastrozole arm, suggesting it might be useful for characterising patients with partial endocrine resistance.

As there is increasing evidence that luminal B biology is a determinant of suboptimal response to endocrine therapy alone, it was hypothesized that the intrinsic subtype could potentially define a subgroup that might derive an increased benefit from combination therapy with pictilisib and anastrozole. We therefore explored the possible interaction between intrinsic subtypes defined by NanoString PAM50 analysis and anti-proliferative response in a subgroup of tumours (n=53) with available pre- and posttreatment RNA. Additional analyses were completed on the entire study population using alternative markers that have been associated with the luminal B phenotype including baseline Ki67 expression, PR expression and tumour grade.

In keeping with our hypothesis, PAM50 analysis showed that patients with Luminal B tumours had a significantly higher anti-proliferative response with combination treatment compared to anastrozole alone [mean Ki67 suppression, 86.5% versus 63.6%; ratio 0.37 (0.18-0.76; p=0.008)], whereas adding pictilisib to anastrozole had no apparent benefit for Luminal A tumours (ratio, 1.01; p=0.98). It is unclear whether this result is more a reflection of the fact that luminal B tumours are partially

endocrine resistant compared to the highly endocrine sensitive luminal A tumours, than a true differential effect of PI3K inhibitors in the respective subtypes.

Defining luminal A and B status through baseline Ki67 expression in accordance with the St Gallen criteria (using a Ki67 cut-off of 14%) provided somewhat contradictory results, demonstrating a significant benefit of combination therapy in patients with Luminal A and Luminal B tumours, using alternative cut-offs of 14% and 20%, respectively. However, much of the benefit in Luminal A tumours seems to be driven by an unexpectedly low Ki67 suppression with anastrozole alone. This is in contrast to other studies (Dowsett et al., 2007). A possible explanation is that the Ki67 suppression results might be less reliable for patients with low baseline expression (<10%) considering the variability in the Ki67 assessment as illustrated in the mean difference of 2.6%-3.9% between the 2 analyses. Some trials therefore exclude patients with a Ki67 baseline expression of <10%. We explored the possibility of an additional analysis, excluding patients with baseline Ki67<10%, but the number were too low for achieving reliable results. Of note, mean baseline Ki67 expression was 15.4% for PAM50 Luminal A and 30.7% for PAM50 Luminal B tumours, with 60% of Luminal A tumours showing baseline Ki67 values of >10%, suggesting that this analysis might be less likely affected by possible technical limitations in tumours with low baseline Ki67 levels.

Additional, pre-planned, subset analyses suggest that the effects of pictilisib added to anastrozole are predominantly seen in patients with PR negative and/or grade 3 tumours. Multivariate linear regression analysis demonstrated an increased treatment effect for the pictilisib containing arm in patients with Luminal B cancers

independent of baseline Ki67 expression, suggesting an impact of molecular subtype on the response to pictilisib independent of baseline proliferation. Overall, these findings are supportive of an association between luminal subtype, insensitivity to endocrine therapies and response to treatment with a pan-PI3K inhibitor, which have implications for future trial design and therapeutic strategies.

To further evaluate treatment-induced changes in gene expression and protein expression/phosphorylation, we used RPPAs and Nanostring gene expression analysis in subsets of patients. All the samples used for the gene expression study were serial sections from the same tumour that were assessed for Ki-67. The performance of the Nanostring platform has been shown to be comparable when using FFPE tissue to snap-frozen (Veldman-Jones et al., 2015). The platform is more sensitive than microarrays when investigating samples with potentially degraded mRNA, reporting excellent reproducibility and robustness as there is no amplification bias in the reaction. The platform hybridises to any mRNA present, producing a direct read-out of genes present in the sample (Geiss et al., 2008). The samples used for RPPA analysis were smaller as we utilised fresh frozen tissue collected. Although FFPE samples can be used for RPPA analysis, the variation of tissue handling at different sites warranted that only snap-frozen tissue was used for this analysis (Boellner and Becker, 2015). We were able to demonstrate profound down-regulation of ER-mediated transcription and cell cycle progression. Interestingly, we found no differences in the expression of ER target genes between both study arms, suggesting that the preclinically observed induction of ER target genes by PI3K inhibition requires oestrogen and is therefore not relevant in the context of combined endocrine and PI3K inhibitor therapy.

RPPA analysis focused on key genes involved in the activation of the PI3K pathway and cell cycle. There was substantial downregulation of cell cycle genes in both arms but Cyclin D1 levels were more suppressed in the combination arm consistent with the more substantial anti-proliferative effect demonstrated in the primary Ki67 analysis. Surprisingly, there was no discernible differences between both groups in the expression and/or phosphorylation of PI3K downstream targets Phospho-AKT, pS6 and p4E-BP1. On the other hand, gene expression analysis demonstrated up-regulation of the PI3K regulated genes IRS2 and PIK3IP1, confirming treatment-associated pathway inhibition. It remains unclear whether the lack of a clear effect on PI3K downstream targets has technical reasons or might instead be reflective of the complex biology of the PI3K/AKT pathway. It is well recognised that pAKT levels can change rapidly during the processing of tissue samples. In the OPPORTUNE trial, we therefore defined strict criteria for rapid processing of tissue samples to minimise these effects, but it cannot be excluded that RPPA results have been affected by this.

Previous publications have found that inhibition of PI3K/Akt/mTOR pathways in patients can be associated with a higher risk of infection due to immunosuppression from the function of the immune system rather than the number of immune cells (Rafii et al., 2015). mTOR inhibitors are well known to immune-suppressive. The suppression of PI3K should alter the signalling pathways for both effector and regulatory cell population, with specific inhibition of PI3K subunits suppressing T_{reg} cells (Carnevalli et al., 2018). Thus it would be of interest to determine if how PI3K inhibition will alter the transcription of genes associated with immune function.

We demonstrated that short-term treatment with pictilisib and/or anastrozole has a modest impact on the tumour immune microenvironment. Effects differed between the two treatment groups suggesting that PI3K inhibition has additional effect to endocrine therapy. Most of these effects were modest and the potential clinical implications remain to be determined but the emerging role of immunotherapy in breast cancer makes underlines the importance of further studies in this context.

There are few areas where improvements could be made for the future from the data presented here. This includes recruiting more patients for each arm to enable in-depth subset analysis as well as modifying the inclusion criteria so as to include patients who are also PR-positive which may benefit more from PI3K-inhibition (Krop et al., 2016). Finally, the main drawback of a WOO study is that it only investigates short-term effects and thus, the benefits cannot currently be extrapolated for patients in the long-term.

In summary, the OPPORTUNE trial is one of the first clinical trials to evaluate PI3K inhibitors in early breast cancer. It provided proof-of-concept by demonstrating that addition of the PI3K inhibitor pictilisib significantly increased the anti-proliferative response to anastrozole in ER-positive early breast cancers. The trial also provided important information on the subgroup of patients who might benefit most from combined therapy. We showed that PIK3CA mutations were not predictive of response to PI3K inhibitors (but highlighted potential differences between the mutations subtypes) and provided clinical evidence that Luminal B cancer, PR-

negative cancers and/or high grade tumours have an increased benefit of PI3K inhibition, which is in keeping with preclinical data. These data should guide optimal patient selection for future trials and could be critical for the successful clinical development of this group of agents in early breast cancer.

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